

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 37/02 // C07K 13/00 C12N 15/12, 15/62		A1	(11) International Publication Number: WO 93/09800 (43) International Publication Date: 27 May 1993 (27.05.93)
(21) International Application Number: PCT/US92/09871			(72) Inventors: and
(22) International Filing Date: 13 November 1992 (13.11.92)			(75) Inventors/Applicants (for US only): RUOSLAHTI, Erkki, I. [US/US]; P.O. Box 1054, Rancho Santa Fe, CA 92107 (US). LONGAKER, Michael, T. [US/US]; 337 Parnassus Avenue, San Francisco, CA 94117 (US). WHITBY, David, J. [GB/GB]; Mill Race Cottage, Adel, Leeds LS16 8AH (GB). HARPER, John, R. [US/US]; 2433 Unicorn Street, Carlsbad, CA 92008 (US). PIERSCHBACHER, Michael, D. [US/US]; 766 Amiford Drive, San Diego, CA 92107 (US). BORDER, Wayne, A. [US/US]; 4464 Abinadi Road, Salt Lake City, UT 84124 (US).
(30) Priority data: 792,192 14 November 1991 (14.11.91) US 882,345 13 May 1992 (13.05.92) US			(74) Agents: CAMPBELL, Cathryn, A. et al.; Campbell & Flores, 4370 La Jolla Village Drive, Suite 700, San Diego, CA 92122 (US).
(60) Parent Application or Grant (63) Related by Continuation US 07/882,345 (CIP) Filed on 13 May 1992 (13.05.92)			(81) Designated States: AU, BB, BG, BR, CA, CS, FI, HU, JP, KP, KR, LK, MG, MN, MW, NO, PL, RO, RU, SD, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).
(71) Applicants (for all designated States except US): LA JOLLA CANCER RESEARCH FOUNDATION [US/US]; 10901 North Torrey Pines Road, La Jolla, CA 92037 (US). THE REGENTS OF THE UNIVERSITY OF CALIFORNIA [US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA 94612-3550 (US). THE UNIVERSITY OF UTAH [US/US]; Salt Lake City, UT 84112 (US).			Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: INHIBITORS OF CELL REGULATORY FACTORS AND METHODS FOR PREVENTING OR REDUCING SCARRING

(57) Abstract

The present invention provides a method of inhibiting an activity of a cell regulatory factor comprising contacting the cell regulatory factor with a purified polypeptide, wherein the polypeptide comprises the cell regulatory factor binding domain of a protein and wherein the protein is characterized by a leucine-rich repeat of about 24 amino acids. In a specific embodiment, the present invention relates to the ability of decorin, a 40,000 dalton protein that usually carries a glycosaminoglycan chain, to bind TGF- β . The invention also provides a novel cell regulatory factor designated MRF. Also provided are methods of identifying, detecting and purifying cell regulatory factors and proteins which bind and affect the activity of cell regulatory factors. The present invention further relates to methods for the prevention or reduction of scarring by administering decorin or a functional equivalent of decorin to a wound. The methods are particularly useful for dermal wounds resulting from burns, injuries or surgery. In addition, the present invention includes pharmaceutical compositions containing decorin or its functional equivalent and a pharmaceutically acceptable carrier useful in such methods. Finally, methods for preventing or inhibiting pathological conditions by administering decorin are also provided.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam

Inhibitors of Cell Regulatory Factors and
Methods for Preventing or Reducing Scarring

This invention was made with support of government grants CA 30199, CA 42507 and CA 28896 from the National 5 Cancer Institute. Therefore, the United States government may have rights in the invention.

BACKGROUND OF THE INVENTION

This invention relates to cell biology and more specifically to the control of cell proliferation.

10 Proteoglycans are proteins that carry one or more glycosaminoglycan chains. The known proteoglycans carry out a wide variety of functions and are found in a variety of cellular locations. Many proteoglycans are components of extracellular matrix, where they participate in the 15 assembly of cells and effect the attachment of cells to the matrix.

One of the key functions of the extracellular matrix is the storage and presentation of growth factors to cells. Proteoglycans are important mediators of growth factor 20 binding, and they have been shown to modulate the biological activities of a variety of growth factors through interaction via their glycosaminoglycan moieties as well as their core proteins (Ruoslahti, 1989; Ruoslahti and Yamaguchi, 1991).

25 Growth factors that bind to glycosaminoglycans include acidic and basic FGF (see Burgess and Maciag, 1989), GM-CSF, interleukin-3 (Roberts et al., 1988), pleiotrophin (Li et al., 1990), amphiregulin (Shoyab et al., 1988), HB-EGF (Higashiyama et al., 1991) and platelet factor 4 (Huang et 30 al., 1982), each of which binds avidly to heparin and heparan sulfate. The binding of FGFs to heparin or to heparan sulfate proteoglycans protects the growth factors from proteolytic degradation and is thought to create a matrix-bound growth factor reservoir (Saksela et al., 1988;

Gospodarowicz et al., 1990) from which the growth factor can be released in an active form by partial proteolysis of the proteoglycan core protein or through degradation of the heparan sulfate moiety of the proteoglycans (Saksela and 5 Rifkin, 1990; Ishai-Michaeli et al., 1990). Basic FGF has to be bound to glycosaminoglycan to be able to interact with its signal transduction receptor (Yayon et al., 1991; Rapraeger et al., 1991).

The binding of TGF- β to proteoglycans represents a 10 different type of growth factor-proteoglycan interaction. TGF- β has been demonstrated to bind to the core proteins of at least two proteoglycans. One of these proteoglycans is is decorin, a small interstitial extracellular matrix proteoglycan that can interact with TGF- β via its core 15 protein (Yamaguchi et al., 1990). Decorin, also known as PG-II or PG-40, is a small proteoglycan produced by fibroblasts. Its core protein has a molecular weight of about 40,000 daltons. The core has been sequenced (Krusius and Ruoslahti, Proc. Natl. Acad. Sci. USA 83:7683 (1986); 20 Day et al. Biochem. J. 248:801 (1987), both of which are incorporated herein by reference) and it is known to carry a single glycosaminoglycan chain of a chondroitin sulfate/dermatan sulfate type (Pearson, et al., J. Biol. Chem. 258:15101 (1983), which is incorporated herein by 25 reference). The only previously known function for decorin is binding to type I and type II collagen and its effect on the fibril formation by these collagens (Vogel, et al., Biochem. J. 223:587 (1984); Schmidt et al., J. Cell Biol. 104:1683, (1987)). Decorin (Krusius and Ruoslahti, 1986) 30 is the prototype of a group of proteoglycans characterized by core proteins of ~40 kDa that consist mainly of leucine-rich repeats of 20 to 24 amino acids (Patthy, 1987). So far, four members of this group of proteoglycans have been cloned; in addition to decorin, these are biglycan (Fisher 35 et al., 1989), fibromodulin (Oldberg et al., 1989) and lumican (Blochberger et al., 1992). Decorin and biglycan

are ubiquitous, although they show a quite divergent localization within tissues, with decorin found more in the extracellular matrix of tissues where it is bound to type I collagen (Vogel et al., 1984; Scott, 1986; Brown and Vogel, 1989) and biglycan localized more closely around cells (Bianco et al., 1990). Fibromodulin has a somewhat more restricted distribution with high concentrations in cartilage, tendon and sclera, while low in skin and mineralized bone (Heinegard et al., 1986). Lumican is found mainly in the cornea (Blochberger et al., 1992). Together, these proteins form a superfamily of proteins (Ruoslahti, Ann. Rev. Cell Biol. 4:229, (1988); McFarland et al., Science 245:494 (1989)).

The second type of TGF- β -binding proteoglycan is the type III TGF- β receptor, betaglycan (Segarini and Seyedin et al., 1988; Andres et al., 1989). Betaglycan is a cell membrane proteoglycan (López-Casillas et al., 1991; Wang et al., 1991) that apparently is not involved in the TGF- β signal transduction pathway but may function as a cell-surface TGF- β reservoir presenting TGF- β to its signal transduction receptors.

Transforming growth factor β s (TGF- β) are a family of multi-functional cell regulatory factors produced in various forms by many types of cells (for review see Sporn et al., J. Cell Biol. 105:1039, (1987)). Five different TGF- β 's are known, but the functions of only two, TGF- β 1 and TGF- β 2, have been characterized in any detail. TGF- β 's are the subject of U.S. Patent Nos. 4,863,899; 4,816,561; and 4,742,003 which are incorporated by reference. TGF- β 1 and TGF- β 2 are publicly available through many commercial sources (e.g. R & D Systems, Inc., Minneapolis, MN). In some cells, TGF- β promotes cell proliferation, in others it suppresses proliferation. A marked effect of TGF- β is that it promotes the production of extracellular matrix proteins

and their receptors by cells (for review see Keski-Oja et al., J. Cell Biochem 33:95 (1987); Massague, Cell 49:437 (1987); Roberts and Sporn in "Peptides Growth Factors and Their Receptors" [Springer-Verlag, Heidelberg] (1989)).

5 While TGF- β has many essential cell regulatory functions, improper TGF- β activity can be detrimental to an organism. Since the growth of mesenchyme and proliferation of mesenchymal cells is stimulated by TGF- β , some tumor cells may use TGF- β as an autocrine growth factor.
10 Therefore, if the growth factor activity of TGF- β could be prevented, tumor growth could be controlled. In other cases the inhibition of cell proliferation by TGF- β may be detrimental, in that it may prevent healing of injured tissues. The stimulation of extracellular matrix
15 production by TGF- β is important in situations such as wound healing. However, in some cases the body takes this response too far and an excessive accumulation of extracellular matrix ensues. An example of excessive accumulation of extracellular matrix is glomerulonephritis,
20 a disease with a detrimental involvement of TGF- β .

Thus, there exists a critical need to develop compounds that can modulate the effects of cell regulatory factors such as TGF- β . The present invention satisfies this need and provides related advantages.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting an activity of a cell regulatory factor comprising contacting the cell regulatory factor with a purified 5 polypeptide, wherein the polypeptide comprises a cell regulatory factor binding domain of a protein and wherein the protein is characterized by a leucine-rich repeat of about 24 amino acids. In a specific embodiment, the present invention relates to the ability of decorin, a 10 40,000 dalton protein that usually carries a glycosaminoglycan chain, to bind TGF- β . The invention also provides a novel cell regulatory factor designated Morphology Restoring Factor, (MRF). Also provided are methods of identifying, detecting and purifying cell 15 regulatory factors and proteins which bind and affect the activity of cell regulatory factors.

The present invention further relates to methods for the prevention or reduction of scarring by administering decorin or a functional equivalent of decorin to a wound. 20 The methods are particularly useful for dermal wounds resulting from burns, injuries or surgery. In addition, the present invention includes pharmaceutical compositions containing decorin or its functional equivalent and a pharmaceutically acceptable carrier useful in such methods. 25 Finally, methods for preventing or inhibiting pathological conditions by administering decorin are also provided.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows expression of decorin cDNA containing a mutation of the serine acceptor site to alanine. COS-1 30 cultures were transfected with cDNA coding for wild-type decorin (lane 1), decorin in which the serine-4 residue was replaced by an alanine (lane 2), or decorin in which the

serine-4 residue was replaced by a threonine (lane 3). Immunoprecipitations were performed with an anti-decorin antibody and medium which was labeled with ^{35}S -sulfate (A) or ^3H -leucine (B). Lane 4 shows an immunoprecipitate from 5 mock transfected COS-1 cultures. Arrow indicates top of gel. The numbers indicate $M_r \times 10^{-3}$ for molecular weight standards.

Figure 2 shows binding of $[^{125}\text{I}]\text{-TGF-}\beta 1$ to decorin-Sepharose. Figure 2A shows fractionation of $[^{125}\text{I}]\text{-TGF-}\beta 1$ 10 by decorin-Sepharose affinity chromatography. $[^{125}\text{I}]\text{-TGF-}\beta 1$ (5×10^5 cpm) was incubated in BSA-coated polypropylene tubes with 0.2 ml of packed decorin-Sepharose (●) or gelatin-Sepharose (○) in 2 ml of PBS pH 7.4, containing 1 M NaCl and 0.05% Tween 20. After overnight incubation, the 15 affinity matrices were transferred into BSA-coated disposable columns (Bio Rad) and washed with the binding buffer. Elution was effected first with 3 M NaCl in the binding buffer and then with 8 M urea in the same buffer. Figure 2B shows the analysis of eluents of decorin- 20 Sepharose affinity chromatography by SDS-polyacrylamide gel under nonreducing conditions. Lane 1: the original $[^{125}\text{I}]$ -labeled TGF- $\beta 1$ sample; lanes 2-7: flow through and wash fractions; lanes 8-10: 3 M NaCl fractions; lanes 11-14: 25 8 M urea fractions. Arrows indicate the top and bottom of the 12% separating gel.

Figure 3 shows the inhibition of binding of $[^{125}\text{I}]\text{-TGF-}\beta 1$ to decorin by proteoglycans and their core proteins. Figure 3A shows the competition of $[^{125}\text{I}]\text{-TGF-}\beta 1$ binding to 30 decorin-coated microtiter wells by recombinant decorin (●), decorin isolated from bovine skin (PGII) (■), biglycan isolated from bovine articular cartilage (PGI) (▲), chicken cartilage proteoglycan (○), and BSA (□). Each point represents the mean of duplicate determinants. Figure 3B shows the competition of $[^{125}\text{I}]\text{-TGF-}\beta 1$ binding with 35 chondroitinase ABC-treated proteoglycans and BSA. The

concentrations of competitors were expressed as intact proteoglycan. The symbols are the same as in Figure 3A.

Figure 4 shows neutralization of the growth regulating activity of TGF- β 1 by decorin. Figure 4A shows inhibition of TGF- β 1-induced proliferation of CHO cells by decorin. The [3 H]Thymidine incorporation assay was performed as described in the legend of Figure 1 in the presence of 5 ng/ml of TGF- β 1 and the indicated concentrations of purified decorin (●) or BSA (○). At the concentration used, TGF- β 1 induced a 50% increase of [3 H]thymidine incorporation in the CHO cells. The data represent percent neutralization of this growth stimulation; i.e. [3 H]thymidine incorporation in the absence of either TGF- β 1 or decorin = 0%, incorporation in the presence of TGF- β but not decorin = 100%. Each point shows the mean \pm standard deviation of triplicate samples. Figure 4B shows neutralization of TGF- β 1-induced growth inhibition in MvLu cells by decorin. Assay was performed as in A except that TGF- β 1 was added at 0.5 ng/ml. This concentration of TGF- β 1 induces 50% reduction of [3 H]thymidine incorporation in the MvLu cells. The data represent neutralization of TGF- β -induced growth inhibition; i.e. [3 H]thymidine incorporation in the presence of neither TGF- β or decorin = 100%; incorporation in the presence of TGF- β but not decorin = 0%.

Figure 5A shows separation of growth inhibitory activity from decorin-expressing CHO cells by gel filtration. Serum-free conditioned medium of decorin overexpressor cells was fractionated by DEAE-Sepharose chromatography in a neutral Tris-HCl buffer and fractions containing growth inhibitory activity were pooled, made 4M with guanidine-HCl and fractionated on a Sepharose CL-6B column equilibrated with the same guanidine-HCl solution. The fractions were analyzed for protein content, decorin content, and growth regulatory activities. Elution

positions of marker proteins are indicated by arrows. BSA: bovine serum albumin (Mr=66,000); CA: carbonic anhydrase (Mr=29,000); Cy: cytochrome c (Mr=12,400); Ap: aprotinin (Mr=6,500); TGF: [¹²⁵I]-TGF- β 1 (Mr=25,000).

5 Figure 5B shows identification of the growth stimulatory material from gel filtration as TGF- β 1. The growth stimulatory activity from the late fractions from Sepharose 6B (bar in panel A) was identified by inhibiting the activity with protein A-purified IgG from an anti-TGF- β 10 antiserum. Data represent percent inhibition of growth stimulatory activity in a [³H]thymidine incorporation assay. Each point shows the mean \pm standard deviation of triplicate determinations. Anti-TGF- β 1 (●), normal rabbit IgG (○).

15 Figure 6 shows micrographs demonstrating a decorin-binding cell regulatory activity that is not suppressed by antibodies to TGF- β 1.

20 Figure 7 shows that decorin inhibits the binding of [¹²⁵I]-TGF- β to Type III TGF- β receptor (β glycan) on HepG2 cells. Figure 7a shows the non-reduced lysate of HepG2 cells resolved on 4-12% SDS-PAGE. Figure 7b shows the reduced lysate resolved on 4-12% SDS-PAGE. The reduction of intensity of β glycan band (approximately 300 kDa) and uncross-linked band (free TGF- β , 25 kDa) in the presence of decorin (10,000 x molar excess) is shown.

25 Figure 8 shows that decorin inhibits the binding of [¹²⁵I]-TGF- β to Type III TGF- β receptor on MG-63 cells. Figure 8a shows the resolution of the lysate on 4-12% SDS-PAGE under non-reduced conditions, while Figure 8b shows the results under reduced conditions.

30 Figure 9 shows that decorin (DC-9, DC-12) and biglycan inhibit the binding of [¹²⁵I]-TGF- β to immobilized decorin.

Figure 10 shows the concentration dependence of decorin inhibition of [¹²⁵I]-TGF- β binding to HepG2 cells.

Figure 11 shows the amino acid sequence of human fibromodulin deduced from cDNA. The human fibromodulin sequence is shown aligned with the amino acid sequences of 5 bovine fibromodulin (Oldberg et al., 1989), human decorin (Krusius and Ruoslahti, 1986) and human biglycan (Fisher et al., 1989). A star marks the sequence position where the NH₂-termini of the proteoglycan core proteins lacking their 10 predicted signal sequences were fused to *E. coli*-maltose binding protein (MBP) with two additional amino acids, glycine and serine, added at the linkage site. Identical amino acids are boxed.

Figure 12 shows the construction of prokaryotic 15 expression vector for proteoglycan fusion proteins. The parent vector pQE-8 was modified by insertion of a BglII/BamHI-MBP fragment. This fragment also included a factor Xa protease cleavage site and provided a unique BamHI cloning site for introduction of the proteoglycan 20 core protein inserts. RBS = ribosomal binding site; 6xHis = coding sequence for consecutive six histidines; MBP = coding sequence for *E. coli*-maltose binding protein; to = transcriptional terminator 'to' of phage lambda (Schwarz et al., 1987); cat = promotor-free gene for chloramphenicol 25 acetyltransferase; T1 = transcriptional terminator T1 of the *E. coli* rrnB operon (Brosius et al., 1981).

Figure 13 shows the analyses by gel electrophoresis of purified recombinant proteoglycan core fusion proteins. Each purified protein (1 μ g/well) was loaded on a 4-20% 30 NaDODSO₄-polyacrylamide gel. After electrophoresis under non-reducing conditions, the gel was stained with Coomassie blue R-250. A = maltose binding protein; B = MBP-biglycan; C = MBP-decorin; D = MBP-fibromodulin. The sizes (kDa) of molecular weight marker proteins are indicated.

Figure 14 shows the binding of radiolabeled proteoglycan fusion proteins and MBP to microtiter wells coated with TGF- β 1. TGF- β 1 was used in the indicated concentrations (75 μ l/well) to coat microtiter wells. The 5 wells were incubated with 125 I-labeled MBP-biglycan (■), MBP-decorin (●), MBP-fibromodulin (▲) or MBP (◆). Constant amounts (~50,000 cpm/well, specific activities 2300-2800 Ci/mmol) of the labeled proteins were added to the TGF- β 1-coated wells (total volume 100 μ l). After incubation for 10 6 hours at 37°C, the wells were washed four times. TGF- β 1-binding was determined by counting the entire wells in a gamma counter and is expressed (\pm S.D.) as percent of the total amount of labeled proteins added to the wells.

Figure 15 shows the specificity of proteoglycan core 15 protein binding to TGF- β 1. Microtiter wells were coated with the indicated proteins (75 μ l/well, 3 μ g/ml). 125 I-MBP-biglycan (hatched bars), 125 I-MBP-decorin (solid bars) or 125 I-MBP-fibromodulin (cross-hatched bars) were added to the wells (total volume 100 μ l). After incubation for 6 hours 20 at 37°C, the wells were washed three times and counted in a gamma counter. Binding (\pm S.D.) is expressed as percent of the total amount of labeled proteins added to the wells.

Figure 16 shows the time-course of MBP-biglycan binding to TGF- β 1. 125 I-MBP-biglycan was added to TGF- β 1 25 coated wells (75 μ l, 1 μ g/ml) at 4°C (●) or 37°C (■), respectively. After the indicated time-periods, the wells were washed three times and counted in a gamma counter. Binding (\pm S.D.) is expressed as percent of the total amount of 125 I-MBP-biglycan added.

30 Figure 17 shows the inhibition of the binding of biglycan fusion protein to TGF- β 1 by proteoglycan fusion proteins and intact proteoglycans. Binding of 125 I-MBP-biglycan to TGF- β 1 was measured in the presence of the indicated concentrations of (A) unlabeled MBP-BG (■), MBP-

DEC (●), MBP-FM (▲) or MBP (♦) or (B) purified biglycan (■), decorin (●) or fibromodulin (▲). After incubation of 6 hours at 37°C, the wells were washed three times and counted in a gamma counter. Binding (±S.D.) is expressed 5 as percent of radiolabel bound in the absence of competitor.

Figure 18 shows the competition for the binding of radiolabeled TGF- β 1, - β 2 and - β 3 to microtiter wells coated with biglycan fusion protein. The binding of 125 I-labeled 10 TGF- β 1 (solid bars), TGF- β 2 (hatched bars) or TGF- β 3 (open bars) (50,000 cpm/well, specific activities 5,000 to 7,000 Ci/mmol) to surface-bound MBP-biglycan (coating concentration 10 μ g/ml. 75 μ l/well) was studied in the absence (control) or presence of unlabeled MBP-BG, MB-DEC, 15 MBP-FM, MBP, biglycan, decorin or fibromodulin (1 μ M). Binding was corrected for nonspecific binding as is expressed as percent (±S.D.) of the total amount of labeled TGF- β 1, 2 or 3 that was added to the wells.

Figure 19 shows the competition for the binding of 20 labeled TGF- β 1 to MvLu cells by proteoglycan fusion proteins. (A) Subconfluent cultures of MvLu mink lung cells cultured in 48-well plates were incubated with 125 I-TGF- β 1 (100 pM) in the presence (n.s.) or absence (B₀) of unlabeled TGF- β 1 (20 nM) or the indicated concentrations of 25 proteoglycan fusion proteins in a total volume of 100 μ l. After incubation for 4 hours at 4°C, the cells were washed four times. The cells were then solubilized for 40 min in 1% Triton-X 100 and assayed for radioactivity in a gamma counter. Binding (±S.D., n=3) is expressed as percent of 30 the total amount of 125 I-TGF- β 1 that was added. (B) Mink lung cells were incubated with 125 I-TGF- β 1 (100 pM) in the absence or presence of unlabeled TGF- β (20 nM) or MBP-fusion proteins (3 μ M) in 24-well plates. After incubation for 4 hours at 4°C, the cells were treated with the cross- 35 linker disuccinimidyl suberate and analyzed by NaDODSO₄-PAGE

and autoradiography. Binding in the absence of competitor (a), with TGF- β 1 (b), MBP-BG (c), MBP-DEC (d), MBP-FM (e) or MBP (f). The positions of pre-stained marker proteins are indicated. The positions of the TGF- β type I and type II receptors and of betaglycan (β -G) are indicated. Arrows point to the receptors and betaglycan (β -G).

DETAILED DESCRIPTION OF THE INVENTION

Increased TGF- β production has been found to be an important element in a number of fibrotic diseases that are characterized by an accumulation of extracellular matrix components (Border and Ruoslahti, 1992). Besides fibronectin, collagens, and tenascin (Ignatza and Massague, 1986; Varga et al., 1987; Pearson et al., 1988), TGF- β also upregulates the expression of proteoglycans (Bassols and Massague, 1988). In mesangial cells both decorin and biglycan can increase as much as 50-fold after induction by TGF- β (Border et al., 1990a), whereas in fibroblasts only biglycan seems to be elevated (Romaris et al., 1992; Kahari et al., 1991). Fibromodulin has not been studied in this regard. TGF- β plays a pivotal role in the pathogenesis of experimentally induced glomerulonephritis, the most critical manifestation of which is the accumulation of extracellular matrix in the glomeruli (Border et al., 1990). A recent study shows that injection of recombinant decorin into glomerulonephritic rats can suppress the matrix accumulation (Border et al., 1992). The present invention indicates that fibromodulin can be even more effective in that situation. The TGF- β neutralizing activities of the decorin-type proteoglycans indicates that new types of therapeutics can be developed based on these molecules.

The invention provides a method of inhibiting an activity of a cell regulatory factor comprising contacting

the cell regulatory factor with a purified polypeptide, wherein the polypeptide comprises the cell regulatory factor binding domain of a protein and wherein the protein is characterized by a leucine-rich repeat of about 24 amino acids. Since diseases such as cancer result from uncontrolled cell proliferation, the invention can be used to treat such diseases.

By "cell regulatory factor" is meant a molecule which can regulate an activity of a cell. The cell regulatory factors are generally proteins which bind cell surface receptors and include growth factors. Examples of cell regulatory factors include the five TGF- β 's, platelet-derived growth factor, epidermal growth factor, insulin like growth factor I and II, fibroblast growth factor, interleukin-2, nerve growth factor, hemopoietic cell growth factors (IL-3, GM-CSF, M-CSF, G-CSF, erythropoietin) and the newly discovered Morphology Restoring Factor, hereinafter "MRF". Different regulatory factors can be bound by different proteins which can affect the regulatory factor's activity. For example, TGF- β 1 is bound by decorin, biglycan and fibromodulin, and MRF is bound by decorin.

By "cell regulatory factor binding domain" is meant the fragment of a protein which binds to the cell regulatory factor. While the specific examples set forth herein utilize proteins, it is understood that a protein fragment which retains the binding activity is included within the scope of the invention. Fragments which retain such activity can be recognized by their ability to competitively inhibit the binding of, for example, decorin to TGF- β , or of other polypeptides containing leucine-rich repeats to their cognate growth factors. As an example, fragments can be obtained by digestion of the native polypeptide or by synthesis of fragments based on the known amino acid sequence. Such fragments can then be used in a

competitive assay to determine whether they retain binding activity. For example, decorin can be attached to an affinity matrix, as by the method of Example II. Labelled TGF- β , and the fragment in question can then be contacted 5 with the affinity matrix and the amount of TGF- β bound thereto determined.

As used herein, "decorin" refers to a proteoglycan having substantially the structural characteristics attributed to it in Krusius and Ruoslahti, supra. Human 10 fibroblast decorin has substantially the amino acid sequence presented in Krusius and Ruoslahti, supra. "Decorin" refers both to the native composition and to modifications thereof which substantially retain the functional characteristics. Decorin core protein refers to 15 decorin that no longer is substantially substituted with glycosaminoglycan and is included in the definition of decorin. Decorin can be rendered glycosaminoglycan-free by mutation or other means, such as by producing recombinant decorin in cells incapable of attaching glycosaminoglycan 20 chains to a core protein.

Functional equivalents of decorin include modifications of decorin that retain its functional characteristics and molecules that are homologous to decorin, such as biglycan and fibromodulin, for example, 25 that have the similar functional activity of decorin. Modifications can include, for example, the addition of one or more side chains that do not interfere with the functional activity of the decorin core protein.

Since the regulatory factor binding proteins each 30 contain leucine-rich repeats of about 24 amino acids which can constitute 80% of the protein, it is likely that the fragments which retain the binding activity occur in the leucine-rich repeats. However, it is possible the binding activity resides in the carboxy-terminal amino acids or the

junction of the repeats and the carboxy terminal amino acids.

The invention teaches a general method whereby one skilled in the art can identify proteins which can bind to cell regulatory factors or identify cell regulatory factors which bind to a certain family of proteins. The invention also teaches a general method whereby these novel proteins or known existing proteins can be assayed to determine if they affect an activity of a cell regulatory factor.

5 Specifically, the invention teaches the discovery that decorin and biglycan bind TGF- β s and MRF and that such binding can inhibit the cell regulatory functions of TGF- β s. Further, both decorin and biglycan are about 80% homologous and contain a leucine-rich repeat of about 24

10 amino acids in which the arrangement of the leucine residues is conserved. As defined each repeat generally contains at least two leucine residues and can contain five or more. These proteoglycans are thus considered members

15 of the same protein family. See Ruoslahti, supra, Fisher et al., J. Biol. Chem., 264:4571-4576 (1989) and Patthy, J. Mol. Biol., 198:567-577 (1987), all of which are incorporated by reference. Other known or later discovered proteins having this leucine-rich repeat, i.e., fibromodulin, would be expected to have a similar cell

20 regulatory activity. The ability of such proteins to bind cell regulatory factors could easily be tested, for example by affinity chromatography or microtiter assay as set forth in Example II, using known cell regulatory factors, such as TGF- β s. Alternatively, any later discovered cell

25 regulatory factor could be tested, for example by affinity chromatography using one or more regulatory factor binding proteins. Once it is determined that such binding occurs, the effect of the binding on the activity of all regulatory factors can be determined by methods such as growth assays

30 as set forth in Example III. Moreover, one skilled in the art could simply substitute a novel cell regulatory factor

35

for a TGF- β or a novel leucine-rich repeat protein for decorin or biglycan in the Examples to determine their activities. Thus, the invention provides general methods to identify and test novel cell regulatory factors and 5 proteins which affect the activity of these factors.

The invention also provides a novel purified compound comprising a cell regulatory factor attached to a purified polypeptide wherein the polypeptide comprises the cell regulatory factor binding domain of a protein and the 10 protein is characterized by a leucine-rich repeat of about 24 amino acids.

The invention further provides a novel purified protein, designated MRF, having a molecular weight of about 20 kd, which can be isolated from CHO cells, copurifies 15 with decorin under nondissociating conditions, separates from decorin under dissociating conditions, changes the morphology of transformed 3T3 cells, and has an activity which is not inhibited with anti-TGF- β 1 antibody. Additionally, MRF separates from TGF- β 1 in HPLC.

20 The invention still further provides a method of purifying a cell regulatory factor comprising contacting the regulatory factor with a protein which binds the cell regulatory factor and has a leucine-rich repeat of about 24 amino acids and to purify the regulatory factor which 25 becomes bound to the protein. The method can be used, for example, to purify TGF- β 1 by using decorin.

The invention additionally provides a method of 30 treating a pathology caused by a TGF- β -regulated activity comprising contacting the TGF- β with a purified polypeptide, wherein the polypeptide comprises the TGF- β binding domain of a protein and wherein the protein is characterized by a leucine-rich repeat of about 24 amino acids, whereby the pathology-causing activity is prevented

or reduced. While the method is generally applicable, specific examples of pathologies which can be treated include cancer, a fibrotic disease, and glomerulonephritis. In fibrotic cancer, for example, decorin can be used to bind TGF- β , destroying TGF- β 's growth stimulating activity on the cancer cell. Other proliferative pathologies include rheumatoid arthritis, arteriosclerosis, adult respiratory distress syndrome, cirrhosis of the liver, fibrosis of the lungs, post-myocardial infarction, cardiac fibrosis, post-angioplasty restenosis, renal interstitial fibrosis and certain dermal fibrotic conditions such as keloids and scarring.

The present invention also provides a method of preventing the inhibition of a cell regulatory factor. The method comprises contacting a protein which inhibits an activity of a cell regulator factor with a molecule which inhibits the activity of the protein. For example, decorin could be bound by a molecule, such as an antibody, which prevents decorin from binding TGF- β s, thus preventing decorin from inhibiting the TGF- β s' activity. Thus, the TGF- β s wound healing activity could be promoted by binding TGF- β 1 inhibitors.

In addition, decorin has been found to inhibit the binding of TGF- β s to their receptors. Figures 7, 8 and 10 show the results of these studies in which cells bearing TGF- β receptors (betaglycan) were incubated with TGF- β in the presence and absence of decorin.

The present invention further relates to methods for the prevention or reduction of scarring by administering decorin or a functional equivalent of decorin to a wound. Dermal scarring is a process, following a variety of dermal injuries, that results in the excessive accumulation of fibrous tissue comprising collagen, fibronectin, and proteoglycans. The induction of fibrous matrix

accumulation is a result of growth factor release at the wound site by platelets and inflammatory cells. The principal growth factor believed to induce the deposition of fibrous scar tissue is transforming growth factor- β 5 (TGF- β). Decorin binds and neutralizes a variety of biological functions of TGF- β , including the induction of extracellular matrix. Due to the lack of elastic property of this fibrous extracellular matrix, the scar tissue resulting from a severe dermal injury often impairs 10 essential tissue function and can result in an unsightly scar.

The advantage of using decorin or a functional equivalent, such as biglycan or fibromodulin, in the methods of the present invention is that it is a normal 15 human protein and is believed to be involved in the natural TGF- β regulatory pathway. Thus, decorin can be used to prevent or reduce dermal scarring resulting from burn injuries, other invasive skin injuries, and cosmetic or reconstructive surgery.

20 Decorin-treated wounds have been found to exhibit essentially no detectable scarring compared to control wounds not treated with decorin. The TGF- β -induced scarring process has been shown to be unique to adults and third trimester human fetuses, but is essentially absent in 25 fetuses during the first two trimesters. The absence of scarring in fetal wounds has been correlated with the absence of TGF- β in the wound bed. In contrast, the wound bed of adult tissue is heavily deposited with TGF- β and the fully healed wound is replaced by a reddened, furrowed scar 30 containing extensively fibrous, collagenous matrix. The decorin-treated wounds were histologically normal and resembled fetal wounds in the first two trimesters.

In addition, the present invention further relates to a pharmaceutical composition containing decorin or its

functional equivalent, such as biglycan or fibromodulin, and a pharmaceutically acceptable carrier useful in the above methods. Pharmaceutically acceptable carriers include, for example, hyaluronic acid, and aqueous 5 solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline supplemented with 5% dextrose or human serum albumin, if desired. The pharmaceutical compositions can also include other agents that promote wound healing known to those skilled in the 10 art. Such agents can include, for example, biologically active chemicals and polypeptides, including RGD-containing polypeptides attached to a biodegradable polymer as described in PCT WO 90/06767 published on June 28, 1990, and incorporated herein by reference. Such polypeptides 15 can be attached to polymers by any means known in the art, including covalent or ionic binding, for example.

It is understood that modifications which do not substantially affect the activity of the various molecules of this invention including TGF- β , MRF, decorin, biglycan 20 and fibromodulin are also included within the definition of those molecules. It is also understood that the core proteins of decorin, biglycan and fibromodulin are also included within the definition of those molecules.

The following examples are intended to illustrate but 25 not limit the invention.

EXAMPLE I

EXPRESSION AND PURIFICATION OF RECOMBINANT DECORIN AND DECORIN CORE PROTEIN

Expression System

30 The 1.8 kb full-length decorin cDNA described in Krusius and Ruoslahti, Proc. Natl. Acad. Sci. USA 83:7683 (1986), which is incorporated herein by reference, was used

for the construction of decorin expression vectors. For the expression of decorin core protein, cDNA was mutagenized so the fourth codon, TCT, coding for serine, was changed to ACT coding for threonine, or GCT coding for 5 alanine. This was engineered by site-directed mutagenesis according to the method of Kunkel, Proc. Natl. Acad. Sci USA 82:488 (1985), which is incorporated herein by reference. The presence of the appropriate mutation was verified by DNA sequencing.

10 The mammalian expression vectors pSV2-decorin and pSV2-decorin/CP-thr4 core protein were constructed by ligating the decorin cDNA or the mutagenized decorin cDNA into 3.4 kb HindIII-Bam HI fragment of pSV2 (Mulligan and Berg, Science 209:1423 (1980), which is incorporated herein 15 by reference).

Dihydrofolate reductase (dhfr)-negative CHO cells (CHO-DG44) were cotransfected with pSV2-decorin or pSV2-decorin/CP and pSV2dhfr by the calcium phosphate coprecipitation method. The CHO-DG44 cells transfected 20 with pSV2-decorin are deposited with the American Type Culture Collection under Accession Number ATCC No. CRL 10332. The transfected cells were cultured in nucleoside-minus alpha-modified minimal essential medium (α -MEM), (GIBCO, Long Island) supplemented with 9% dialyzed fetal 25 calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. Colonies arising from transfected cells were picked using cloning cylinders, expanded and checked for the expression of decorin by immunoprecipitation from 35 SO₄-labeled culture supernatants. 30 Clones expressing a substantial amount of decorin were then subjected to gene amplification by stepwise increasing concentration of methotrexate (MTX) up to 0.64 μ M (Kaufman and Sharp, J. Mol. Biol. 159:601 (1982), which is incorporated herein by reference). All the amplified cell 35 lines were cloned either by limiting dilution or by picking

single MTX resistant colonies. Stock cultures of these established cell lines were kept in MTX-containing medium. Before use in protein production, cells were subcultured in MTX-minus medium from stock cultures and passed at least 5 once in this medium to eliminate the possible MTX effects.

Alternatively, the core protein was expressed in COS-1 cells as described in Adams and Rose, Cell 41:1007, (1985), which is incorporated herein by reference. Briefly, 6-well multiwell plates were seeded with 3-5x10⁵ cells per 9.6 cm² 10 growth area and allowed to attach and grow for 24 hours. Cultures were transfected with plasmid DNA when they were 50-70% confluent. Cell layers were washed briefly with Tris buffered saline (TBS) containing 50 mM Tris, 150 mM NaCl pH 7.2, supplemented with 1 mM CaCl₂, and 0.5 mM MgCl₂, 15 at 37°C to prevent detachment. The wells were incubated for 30 minutes at 37°C with 1 ml of the above solution containing 2 µg of closed circular plasmid DNA and 0.5 mg/ml DEAE-Dextran (Sigma) of average molecular mass of 500,000. As a control, cultures were transfected with the 20 pSV2 expression plasmid lacking any decorin insert or mock transfected with no DNA. Cultures were then incubated for 3 hours at 37°C with Dulbecco's Modified Eagle's medium (Irvine Scientific) containing 10% fetal calf serum and 100 µM chloroquine (Sigma), after removing the DNA/TBS/DEAE- 25 Dextran solution and rinsing the wells with TBS. The cell layers were then rinsed twice and cultured in the above medium, lacking any chloroquine, for approximately 36 hours. WI38 human embryonic lung fibroblasts were routinely cultured in the same medium.

30 COS-1 cultures were radiolabeled 36-48 hours after transfection with the plasmid DNAs. All radiolabeled metabolic precursors were purchased from New England Nuclear (Boston, MA). The isotopes used were ³⁵S-sulfate (460 mCi/ml), L-[3,4,5-³H(N)] -leucine (140 Ci/ml) and L- 35 [¹⁴C(U)] - amino acid mixture (product number 445E).

Cultures were labeled for 24 hours in Ham's F-12 medium (GIBCO Labs), supplemented with 10% dialyzed fetal calf serum, 2 mM glutamine and 1 mM pyruvic acid, and containing 200 μ Ci/ml 35 S-sulfate or 3 H-leucine, or 10 μ Ci/ml of the 5 14 C-amino acid mixture. The medium was collected, supplemented with 5 mM EDTA, 0.5 mM phenylmethylsulfonylfluoride, 0.04 mg/ml aprotinin and 1 μ g/ml pepstatin to inhibit protease activity, freed of cellular debris by centrifugation for 20 minutes at 2,000 10 x G and stored at -20°C. Cell extracts were prepared by rinsing the cell layers with TBS and then scraping with a rubber policeman into 1 ml/well of ice cold cell lysis buffer: 0.05 M Tris-HCl, 0.5 M NaCl, 0.1% BSA, 1% NP-40, 0.5% Triton X-100, 0.1% SDS, pH 8.3. The cell extracts 15 were clarified by centrifugation for 1.5 hours at 13,000 x G at 4°C.

Rabbit antiserum was prepared against a synthetic peptide based on the first 15 residues of the mature form of the human decorin core protein (Asp-Glu-Ala-Ser-Gly-Ile- 20 Gly-Pro-Glu-Val-Pro-Asp-Asp-Arg-Asp). The synthetic peptide and the antiserum against it have been described elsewhere (Krusius and Ruoslahti, 1986 supra.) Briefly, the peptide was synthesized with a solid phase peptide synthesizer (Applied Biosystems, Foster City, CA) by using 25 the chemistry suggested by the manufacturer. The peptide was coupled to keyhole limpet hemocyanin by using N-succinimidyl 3-(2-pyridyldithio) propionate (Pharmacia Fine Chemicals, Piscataway, NJ) according to the manufacturer's instructions. The resulting conjugates were emulsified in 30 Freund's complete adjuvant and injected into rabbits. Further injections of conjugate in Freund's incomplete adjuvant were given after one, two and three months. The dose of each injection was equivalent to 0.6 mg of peptide. Blood was collected 10 days after the third and fourth 35 injection. The antisera were tested against the glutaraldehyde-cross linked peptides and isolated decorin

in ELISA (Engvall, Meth. Enzymol. 70:419-439 (1980)), in immunoprecipitation and immunoblotting, and by staining cells in immunofluorescence, as is well known in the art.

Immunoprecipitations were performed by adding 20 μ l of 5 antiserum to the conditioned medium or cell extract collected from duplicate wells and then mixing overnight at 4°C. Immunocomplexes were isolated by incubations for 2 hours at 4°C with 20 μ l of packed Protein A-agarose (Sigma). The beads were washed with the cell lysis buffer, 10 with three tube changes, and then washed twice with phosphate-buffered saline prior to boiling in gel electrophoresis sample buffer containing 10% mercaptoethanol. Immunoprecipitated proteins were 15 separated by SDS-PAGE in 7.5-20% gradient gels or 7.5% non-gradient gels as is well known in the art. Fluorography was performed by using Enlightning (New England Nuclear) with intensification screens. Typical exposure times were for 7-10 days at -70°C. Autoradiographs were scanned with an LKB Ultroscan XL Enhanced Laser Densitometer to compare 20 the relative intensities and mobilities of the proteoglycan bands.

SDS-PAGE analysis of cell extracts and culture medium from COS-1 cells transfected with the decorin-pSV2 construct and metabolically radiolabeled with 35 S-sulfate 25 revealed a sulfated band that was not present in mock-transfected cells. Immunoprecipitation with the antiserum raised against a synthetic peptide derived from the decorin core protein showed that the new band was decorin.

Expression of the construct mutated such that the 30 serine residue which is normally substituted with a glycosaminoglycan (serine-4) was replaced by a threonine residue by SDS-PAGE revealed only about 10% of the level of proteoglycan obtained with the wild-type construct. The rest of the immunoreactive material migrated at the

position of free core protein.

The alanine-mutated cDNA construct when expressed and analyzed in a similar manner yielded only core protein and no proteoglycan form of decorin. Figure 1 shows the 5 expression of decorin (lanes 1) and its threonine-4 (lanes 3) and alanine-4 (lanes 2) mutated core proteins expressed in COS cell transfectants. $^{35}\text{SO}_4$ -labeled (A) and ^3H -leucine labeled (B) culture supernatants were immunoprecipitated with rabbit antipeptide antiserum prepared against the NH_2 -10 terminus of human decorin.

Purification of Decorin and Decorin Core Protein from Spent Culture Media

Cells transfected with pSV2-decorin vector and amplified as described above and in Yamaguchi and 15 Ruoslahti, *Nature* 36:244-246 (1988), which is incorporated herein by reference, were grown to 90% confluence in 8 175 cm^2 culture flasks in nucleoside minus α -MEM supplemented with 9% dialyzed fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. At 90% 20 confluence culture media was changed to 25 ml per flask of nucleoside-free α -MEM supplemented with 6% dialyzed fetal calf serum which had been passed through a DEAE Sepharose Fast Flow column (Pharmacia) equilibrated with 0.25 M NaCl in 0.05 M phosphate buffer, pH 7.4. Cells were cultured 25 for 3 days, spent media was collected and immediately made to 0.5 mM phenylmethylsulfonyl fluoride, 1 $\mu\text{g}/\text{ml}$ pepstatin, 0.04 mg/ml aprotinin and 5 mM EDTA.

Four hundred milliliters of the spent media were first passed through gelatin-Sepharose to remove fibronectin and 30 materials which would bind to Sepharose. The flow-through fraction was then mixed with DEAE-Sepharose pre-equilibrated in 50 mM Tris/HCl, pH 7.4, plus 0.2 M NaCl and batch absorbed overnight at 4° C with gentle mixing. The

slurry was poured into a 1.6 x 24 cm column, washed extensively with 50 mM Tris/HCl, pH 7.4, containing 0.2 M NaCl and eluted with 0.2 M - 0.8 M linear gradient of NaCl in 50 mM Tris/HCl, pH 7.4. Decorin concentration was 5 determined by competitive ELISA as described in Yamaguchi and Ruoslahti, supra. The fractions containing decorin were pooled and further fractionated on a Sephadex gel filtration column equilibrated with 8 M urea in the Tris-HCl buffer. Fractions containing decorin were collected.

10 The core protein is purified from cloned cell lines transfected with the pSV2-decorin/CP vector or the vector containing the alanine-mutated cDNA and amplified as described above. These cells are grown to confluence as described above. At confluence the cell monolayer is 15 washed four times with serum-free medium and incubated in α MEM supplemented with 2 mM glutamine for 2 hours. This spent medium is discarded. Cells are then incubated with α MEM supplemented with 2 mM glutamine for 24 hours and the spent media are collected and immediately made to 0.5 mM 20 phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin, 0.04 mg/ml aprotinin and 5 mM EDTA as serum-free spent media. The spent media are first passed through gelatin-Sepharose and the flow-through fraction is then batch-absorbed to CM-Sepharose Fast Flow (Pharmacia Fine Chemicals, Piscataway, 25 NJ) pre-equilibrated in 50 mM Tris/HCl, pH 7.4 containing 0.1 M NaCl. After overnight incubation at 4°C, the slurry is poured into a column, washed extensively with the pre-equilibration buffer and eluted with 0.1M - 1M linear gradient of NaCl in 50 mM Tris/HCl, pH 7.4. The fractions 30 containing decorin are pooled, dialyzed against 50 mM NH₄HCO₃, and lyophilized. The lyophilized material is dissolved in 50 mM Tris, pH 7.4, containing 8M urea and applied to a Sephadex S-200 column (1.5 X 110 cm). Fractions containing decorin core proteins as revealed by 35 SDS-polyacrylamide electrophoresis are collected and represent purified decorin core protein.

EXAMPLE II
BINDING OF TGF- β TO DECORIN

a. Affinity Chromatography of TGF- β on Decorin-Sepharose

Decorin and gelatin were coupled to cyanogen bromide-
5 activated Sepharose (Sigma) by using 1 mg of protein per ml
of Sepharose matrix according to the manufacturer's
instructions. Commercially obtained TGF- β 1 (Calbiochem, La
Jolla, CA) was 125 I-labelled by the chloramine T method
(Frolik et al., J. Biol. Chem. 259:10995-11000 (1984))
10 which is incorporated herein by reference and the labeled
TGF- β was separated from the unreacted iodine by gel
filtration on Sephadex G-25, equilibrated with phosphate
buffered saline (PBS) containing 0.1% bovine serum albumin
(BSA) (Figure 2). $[^{125}$ I]-TGF- β 1 (5×10^5 cpm) was incubated
15 in BSA-coated polypropylene tubes with 0.2 ml of packed
decorin-Sepharose (●) or gelatin-Sepharose (○) in 2 ml of
PBS pH 7.4, containing 1 M NaCl and 0.05% Tween 20. After
overnight incubation, the affinity matrices were
transferred into BSA-coated disposable columns (Bio Rad)
20 and washed with the binding buffer. Elution was effected
first with 3 M NaCl in the binding buffer and then with 8
M urea in the same buffer. Fractions were collected,
counted for radioactivity in a gamma counter and analyzed
by SDS-PAGE under nonreducing condition using 12% gels.

25 Figure 2A shows the radioactivity profile from the two
columns and the SDS-PAGE analysis of the fractions is shown
in Figure 2B. The TGF- β 1 starting material contains a
major band at 25 kd. This band represents the native TGF- β 1
30 dimer. In addition, there are numerous minor bands in
the preparation. About 20-30% of the radioactivity binds
to the decorin column and elutes with 8 M urea, whereas
only about 2% of the radioactivity is present in the urea-
eluted fraction in the control fractionation performed on
gelatin-Sepharose (Figure 2A). The decorin-Sepharose

nonbound fraction contains all of the minor components and some of the 25 kd TGF- β 1, whereas the bound, urea-eluted fraction contains only TGF- β 1 (Figure 2B). These results show that TGF- β 1 binds specifically to decorin, since among 5 the various components present in the original TGF- β 1 preparation, only TGF- β 1 bound to the decorin-Sepharose affinity matrix and since there was very little binding to the control gelatin-Sepharose affinity matrix. The TGF- β 1 that did not bind to the decorin-Sepharose column may have 10 been denatured by the iodination. Evidence for this possibility was provided by affinity chromatography of unlabeled TGF- β 1 as described below.

In a second experiment, unlabeled TGF- β 1 180 ng was fractionated on decorin-Sepharose as described above for 15 125 I-TGF- β .

TGF- β 1 (180 ng) was incubated with decorin-Sepharose or BSA-agarose (0.2 ml packed volume) in PBS (pH 7.4) containing 1% BSA. After overnight incubation at 4°C, the resins were washed with 15 ml of the buffer and eluted 20 first with 5 ml of 3 M NaCl in PBS then with 5 ml of PBS containing 8 M urea. Aliquots of each pool were dialyzed against culture medium without serum and assayed for the inhibition of [3 H]thymidine incorporation in MvLu cells (Example III). The amounts of TGF- β 1 in each pool were 25 calculated from the standard curve of [3 H]thymidine incorporation obtained from a parallel experiment with known concentration of TGF- β 1. The results show that the TGF- β 1 bound essentially quantitatively to the decorin column, whereas there was little binding to the control 30 column (Table 1). The partial recovery of the TGF- β 1 activity may be due to loss of TGF- β 1 in the dialyses.

TABLE I

Decorin-Sepharose affinity chromatography of nonlabeled TGF- β 1 monitored by growth inhibition assay in MvLu cells.

5	Elution	TGF- β 1 (ng)	
		Decorin-Sepharose	BSA-Sepharose
	Flow through & wash	2.7 (2.3%)	82.0 (93.9%)
	3 M NaCl	2.2 (1.8%)	1.3 (1.5%)
10	8 M Urea	116.0 (95.9%)	4.0 (4.6%)

b. Binding of TGF- β 1 to Decorin in a Microtiter Assay: Inhibition by Core Protein and Biglycan

The binding of TGF- β 1 to decorin was also examined in a microtiter binding assay. To perform the assay, the 15 wells of a 96-well microtiter plate were coated overnight with 2 μ g/ml of recombinant decorin in 0.1 M sodium carbonate buffer, pH 9.5. The wells were washed with PBS containing 0.05% Tween (PBS/Tween) and samples containing 5 x 10⁴ cpm of [¹²⁵I]-TGF- β 1 and various concentrations of 20 competitors in PBS/Tween were added to each well. The plates were then incubated at 37°C for 4 hours (at 4°C overnight in experiments with chondroitinase ABC-digested proteoglycans), washed with PBS/Tween and the bound radioactivity was solubilized with 1% SDS in 0.2 M NaOH. 25 Total binding without competitors was about 4% under the conditions used. Nonspecific binding, determined by adding 100-fold molar excess of unlabeled TGF- β 1 over the labeled TGF- β 1 to the incubation mixture, was about 13% of total binding. This assay was also used to study the ability of 30 other decorin preparations and related proteins to compete with the interaction.

Completion of the decorin binding was examined with

the following proteins (Figure 3; symbols are indicated in the section of BRIEF DESCRIPTION OF THE FIGURES): (1) Decorin isolated from bovine skin (PGII), (2) biglycan isolated from bovine articular cartilage (PGI) (both PGI 5 and PGII were obtained from Dr. Lawrence Rosenberg, Montefiore Medical Center, N.Y.; and described in Rosenberg et al., J. Biol. Chem. 250:6304-6313, (1985), incorporated by reference herein), and (3) chicken cartilage proteoglycan (provided by Dr. Paul Goetinck, La Jolla 10 Cancer Research Foundation, La Jolla, CA, and described in Goetinck, P.F., in The Glycoconjugates, Vol. III, Horwitz, M.I., Editor, pp. 197-217, Academic Press, NY).

For the preparation of core proteins, proteoglycans were digested with chondroitinase ABC (Seikagaku, Tokyo, 15 Japan) by incubating 500 µg of proteoglycan with 0.8 units of chondroitinase ABC in 250 µl of 0.1 M Tris/Cl, pH 8.0, 30 mM sodium acetate, 2 mM PMSF, 10 mM N-ethylmaleimide, 10 mM EDTA, and 0.36 mM pepstatin for 1 hour at 37°C. Recombinant decorin and decorin isolated from bovine skin 20 (PGII) inhibited the binding of [¹²⁵I]-TGF- β 1, as expected (Figure 3A). Biglycan isolated from bovine articular cartilage was as effective an inhibitor as decorin. Since chicken cartilage proteoglycan, which carries many chondroitin sulfate chains, did not show any inhibition, 25 the effect of decorin and biglycan is unlikely to be due to glycosaminoglycans. Bovine serum albumin did not show any inhibition. This notion was further supported by competition experiments with the mutated decorin core protein (not shown) and chondroitinase ABC-digested decorin 30 and biglycan (Figure 3B). Each of these proteins was inhibitory, whereas cartilage proteoglycan core protein was not. The decorin and biglycan core proteins were somewhat more active than the intact proteoglycans. Bovine serum albumin treated with chondroitinase ABC did not show any 35 inhibition. Additional binding experiments showed that [¹²⁵I]-TGF- β 1 bound to microtiter wells coated with biglycan

or its chondroitinase-treated core protein. These results show that TGF- β 1 binds to the core protein of decorin and biglycan and implicates the leucine-rich repeats these proteins share as the potential binding sites.

5

EXAMPLE III

ANALYSIS OF THE EFFECT OF DECORIN ON CELL PROLIFERATION STIMULATED OR INHIBITED BY TGF- β 1

The ability of decorin to modulate the activity of TGF- β 1 was examined in [3 H]thymidine incorporation assays. 10 In one assay, an unamplified CHO cell line transfected only with pSV2dhfr (control cell line A in reference 1, called CHO cells here) was used. The cells were maintained in nucleoside-free alpha-modified minimal essential medium (α -MEM, GIBCO, Long Island, NY) supplemented with 9% dialyzed 15 fetal calf serum (dFCS) and [3 H]thymidine incorporation was assayed as described (Cheifetz et al., Cell 48:409-415 (1987)). TGF- β 1 was added to the CHO cell cultures at 5 ng/ml. At this concentration, it induced a 50% increase of [3 H]thymidine incorporation in these cells. Decorin or BSA 20 was added to the medium at different concentrations. The results are shown in Figure 4A. The data represent percent neutralization of the TGF- β 1-induced growth stimulation, i.e., [3 H]thymidine incorporation, in the absence of either TGF- β 1 or decorin = 0%, incorporation in the presence of 25 TGF- β 1 but not decorin = 100%. Each point shows the mean \pm standard deviation of triplicate samples. Decorin (●) BSA (○).

Decorin neutralized the growth stimulatory activity of TGF- β 1 with a half maximal activity at about 5 μ g/ml. 30 Moreover, additional decorin suppressed the [3 H]-thymidine incorporation below the level observed without any added TGF- β 1, demonstrating that decorin also inhibited TGF- β made by the CHO cells themselves. Both the decorin-expressor and control CHO cells produced an apparently

active TGF- β concentration of about 0.25 ng/ml concentration into their conditioned media as determined by the inhibition of growth of the mink lung epithelial cells. (The assay could be performed without interference from the 5 decorin in the culture media because, as shown below, the effect of TGF- β on the mink cells was not substantially inhibited at the decorin concentrations present in the decorin-producer media.)

Experiments in MvLu mink lung epithelial cells 10 (American Type Culture Collection CCL 64) also revealed an effect by decorin on the activity of TGF- β 1. Figure 4B shows that in these cells, the growth of which is measured by thymidine incorporation, had been suppressed by TGF- β 1. Assay was performed as in Figure 4A, except that TGF- β 1 was 15 added at 0.5 ng/ml. This concentration of TGF- β induces 50% reduction of [3 H]-thymidine incorporation in the MvLu cells. The data represent neutralization of TGF- β -induced growth inhibition; i.e., [3 H]-thymidine incorporation in the presence of neither TGF- β or decorin = 100%; incorporation 20 in the presence of TGF- β but not decorin = 0%.

EXAMPLE IV
NEW DECORIN-BINDING FACTOR THAT CONTROLS CELL SPREADING
AND SATURATION DENSITY

Analysis of the decorin contained in the overexpressor 25 culture media not only uncovered the activities of decorin described above, but also revealed the presence of other decorin-associated growth regulatory activities. The overexpressor media were found to contain a TGF- β -like growth inhibitory activity. This was shown by gel 30 filtration of the DEAE-isolated decorin under dissociating conditions. Serum-free conditioned medium of decorin overexpressor CHO-DG44 cells transfected with decorin cDNA was fractionated by DEAE-Sepharose chromatography in a neutral Tris-HCl buffer and fractions containing growth

inhibitory activity dialyzed against 50 mM NH₄HCO₃, lyophilized and dissolved in 4 M with guanidine-HCl in a sodium acetate buffer, pH 5.9. The dissolved material was fractionated on a 1.5 x 70 cm Sepharose CL-6B column 5 equilibrated with the same guanidine-HCl solution. The fractions were analyzed by SDS-PAGE, decorin ELISA and cell growth assays, all described above. Three protein peaks were obtained. One contained high molecular weight proteins such as fibronectin (m.w. 500,000) and no 10 detectable growth regulatory activities, the second was decorin with the activities described under Example III and the third was a low molecular weight (10,000-30,000-dalton) fraction that had a growth inhibitory activity in the mink cell assay and stimulated the growth of the CHO cells. 15 Figure 5 summarizes these results. Shown are the ability of the gel filtration fractions to affect [³H]-thymidine incorporation by the CHO cells and the concentration of decorin as determined by enzyme immunoassay. Shown also (arrows) are the elution positions of molecular size 20 markers: BSA, bovine serum albumin (Mr=66,000); CA, carbonic anhydrase (Mr=29,000); Cy, cytochrome c (Mr=12,400); AP, aprotinin (Mr=6,500); TGF, [¹²⁵I]-TGF- β 1 (Mr=25,000).

The nature of the growth regulatory activity detected 25 in the low molecular weight fraction was examined with an anti-TGF- β 1 antiserum. The antiserum was prepared against a synthetic peptide from residues 78-109 of the human mature TGF- β 1. Antisera raised by others against a cyclic form of the same peptide, the terminal cysteine residues of 30 which were disulfide-linked, have previously been shown to inhibit the binding of TGF- β 1 to its receptors (Flanders et al., Biochemistry 27:739-746 (1988), incorporated by reference herein). The peptide was synthesized in an Applied Biosystems solid phase peptide synthesizer and 35 purified by HPLC. A rabbit was immunized subcutaneously with 2 mg per injection of the peptide which was mixed with

0.5 mg of methylated BSA (Sigma, St. Louis, MO) and emulsified in Freund's complete adjuvant. The injections were generally given four weeks apart and the rabbit was bled approximately one week after the second and every 5 successive injection. The antisera used in this work has a titer (50% binding) of 1:6,000 in radioimmunoassay, bound to TGF- β 1 in immunoblots.

This antiserum was capable of inhibiting the activity of purified TGF- β 1 on the CHO cells. Moreover, as shown in 10 Figure 5, the antiserum also inhibited the growth stimulatory activity of the low molecular weight fraction as determined by the [3 H]-thymidine incorporation assay on the CHO cells. Increasing concentrations of an IgG fraction prepared from the anti-TGF- β 1 antiserum suppressed 15 the stimulatory effect of the low molecular weight fraction in a concentration-dependent manner (●). IgG from a normal rabbit serum had no effect in the assay (○).

The above result identified the stimulatory factor in the low molecular weight fraction as TGF- β 1. However, TGF- β 1 is not the only active compound in that fraction. Despite the restoration of thymidine incorporation by the 20 anti-TGF- β 1 antibody shown in Figure 5, the cells treated with the low molecular weight fraction were morphologically different from the cells treated with the control IgG or 25 cells treated with antibody alone. This effect was particularly clear when the antibody-treated, low molecular weight fraction was added to cultures of H-ras transformed NIH 3T3 cells (Der et al., Proc. Natl. Acad. Sci. USA 79:3637-3640 (1982)). As shown in Figure 6, cells treated 30 with the low molecular weight fraction and antibody (micrograph in panel B) appeared more spread and contact inhibited than the control cells (micrograph in panel A). This result shows that the CHO cell-derived recombinant 35 decorin is associated with a cell regulatory factor, MRF, distinct from the well characterized TGF- β 's.

Additional evidence that the new factor is distinct from TGF- β 1 came from HPLC experiments. Further separations of the low molecular weight from the Sepharose CL-6B column was done on a Vydac C4 reverse phase column (1 5 x 25 cm, 5 μ m particle size, the Separations Group, Hesperia, CA) in 0.1% trifluoroacetic acid. Bound proteins were eluted with a gradient of acetonitrile (22-40%) and the fractions were assayed for growth-inhibitory activity in the mink lung epithelial cells and MRF activity in H-ras 10 3T3 cells. The result showed that the TGF- β 1 activity eluted at the beginning of the gradient, whereas the MRF activity eluted toward the end of the gradient.

EXAMPLE V
INHIBITION OF TGF- β BINDING

15 A. Cross Linking of [125 I]-TGF- β to HepG2 Cells

About 2.5×10^4 HepG2 cells (human hepatocellular carcinoma, ATCC No. HB 8065) were incubated with 100 pM [125 I]-TGF- β in the presence of recombinant decorin, TGF- β , or α -TGF- β antibody for 2 hours at room temperature. Cells 20 were washed four times prior to suspension in binding buffer (128 mM NaCl, 5 mM KCl, 5 mM Mg_2SO_4 , 1.2 mM CaCl₂, 50 mM HEPES, 2 mg/ml BSA, pH 7.5) containing 0.25 mM disuccinimidyl suberate (DSS) for 15 minutes. Cells were subsequently washed in washing buffer (binding buffer 25 without BSA) containing 150 mM sucrose and lysed before suspension in Laemmli sample buffer, which is known to those skilled in the art, containing SDS. The lysates were resolved on 4-12% SDS-PAGE under reducing and non-reducing conditions. Cross-linked TGF- β was visualized by 30 autoradiography.

Figure 7 shows the results of the studies. Decorin inhibits the binding of TGF- β to β glycan, a TGF- β receptor found on HepG2 cells.

B. Cross Linking of [¹²⁵I]-TGF- β to MG-63 Cells

About 10^5 MG-63 cells (male osteosarcoma, ATCC No. CRL 1427) were incubated with 150 pM [¹²⁵I]-TGF- β in the presence of a recombinant decorin preparation (designated as DC-13) 5 or TGF- β for 2 hours at room temperature. Cells were washed four times in ice cold binding buffer of Example V(A) prior to suspension in binding buffer containing 0.25 mM DSS for 15 minutes. Cells were washed in 250 mM sucrose buffer before lysis in 1% Triton X-100 buffer, containing 10 protease inhibitors. Lysed cells were centrifuged at 12,000 x g to remove nuclei. Equivalent volumes of Laemmli SDS sample buffer were added to each supernatant prior to electrophoresis through 4-12% tris-glycine gels. The cross-linked TGF-225 was visualized by autoradiography.

15 Figure 8 shows the results of the studies. Similar to the above studies with HepG2 cells, decorin also inhibits TGF- β binding to its receptors on the MG-63 cells.

C. Binding Studies of ¹²⁵I-TGF- β to Immobilized Decorin

A 96-well Linbro microtiter plate was coated with 0.5 20 μ g/ml recombinant decorin at 50 μ l/well. The plate was placed in a 37°C incubator overnight and thereafter washed 3 times with 200 μ l PBS (0.15 M NaCl) per well to remove unbound decorin. TGF- β labeled with ¹²⁵I (400 pM, New England Nuclear, Bolton-Hunter Labeled) was pre-incubated 25 with or without competitors in 200 μ l PBS/0.05% Tween-20 for 1 hour, 45 minutes at room temperature. Competitors included recombinant human decorin preparations (DC-9 and DC-12) and biglycan, with MBP as a negative control. DC-9 and DC-12 are different preparations of recombinant human decorin; PT-71 or MBP (maltose-binding protein) is a negative control; and biglycan is recombinant human biglycan.

Fifty μ l/well of the pre-incubated TGF- β mixture or control were added and incubated overnight at 0°C. Following the incubation, 50 μ l of the free TGF- β supernatants were transferred to labeled tubes. The plate 5 was washed 3 times with 0.05% Tween-20 in PBS (200 μ l/well). Reducing sample buffer (2X Laemmli sample buffer) was added at 100 μ l/well and incubated at 37°C for 30 minutes. While gently pulsing the solution, 100 μ l of bound 125 I-TGF- β was removed from each well and transferred 10 into tubes for counting in a gamma counter. The 50 μ l free TGF- β samples were counted in parallel to the 100 μ l bound TGF- β samples to obtain the bound:free ratio. The results of the binding studies with immobilized decorin are summarized in Figure 9.

15 D. Binding of 125 I-TGF- β to HepG2 Cells

About 2.5×10^4 HepG2 cells were incubated with 250 pM $[^{125}$ I]-TGF- β , in the presence of recombinant human decorin (DC-12) or PT-71 (MBP) for 2 hours at room temperature. Cells were washed with the washing buffer of Example V(A) 20 four times before determination of bound CPM.

The results are summarized in Figure 10. Table II provides numerical data for decorin (DC-12) inhibition of TGF- β binding to HepG2 cells. The "% Change" represents the difference in the mean cpm of the test samples compared 25 to the cpm of the medium (negative control). The α -TGF- β antibody inhibits the binding of labeled TGF- β to cells bearing TGF- β receptors and serves as a positive control.

TABLE II
BINDING OF ^{125}I -TGF- β 1 TO HEPG2 CELLS

<u>Treatment</u>	<u>Concentration</u>	CPM	<u>Mean</u>	<u>% Change</u>
Medium*	---	13,899** 13,898 12,529 11,764 12,694 12,448	12,872 \pm 856	---
TGF- β 1	2.5 x 10E-8M	3,092 2,543 2,800	2,812 \pm 275	-78
Anti-TGF- β 1 (R&D)	2.5 x 10E-7M	6,191 4,848 3,839	4,959 \pm 1,180	-61
Decorin (DC-12)	2.5 x 10E-6M	2,745 2,844 1,945	2,511 \pm 493	-80
	2.5 x 10E-7M	4,258 5,914 4,052	4,741 \pm 1,021	-63
	2.5 x 10E-8M	13,596 12,798 11,599	12,664 \pm 1,005	-2
PT-71	2.5 x 10E-6M	11,859 13,129 12,348	12,449 \pm 636	-3
	2.5 x 10E-7M	11,259 11,022 9,343	10,541 \pm 1,045	-18
	2.5 x 10E-8M	10,886 10,778 10,104	10,589 \pm 424	-18

* 25,000 HepG2 cells obtained from subconfluent cultures were incubated with 250 pM ^{125}I -TGF- β 1 and TGF- β , anti TGF- β , decorin, or decorin fragments for 2 hours at room temperature.

** Unbound ^{125}I -TGF- β 1 was separated from bound by washing cells 4x.

EXAMPLE VI
SCARRING STUDIES

Adult mice were incised with paired longitudinal wounds on the shaved dorsal skin. Care was taken to cut 5 through the panniculus down to the skeletal musculature of the dorsal skin. The incisions were treated with a 250 μ l single dose of either 10 mg/ml hyaluronic acid (control), or a decorin (0.5 mg/ml)/hyaluronic acid (10 mg/ml) mixture in TBS. To form the mixture, 0.5 mg/ml of recombinant 10 decorin was mixed with 10 mg/ml of hyaluronic acid. Each mouse had a blinded control and treated incision. The wounds were sutured closed. Following 14 days, the 15 incisions were monitored grossly and harvested for histology. Frozen sections of the control and treated incision sites (4 microns) were analyzed using standard histological procedures with Masson's trichrome to visualize the staining.

The hyaluronic acid control exhibited a typical dermal scar as is seen in normal adult animals, whereas the 20 decorin-treated wounds exhibited no detectable scar and were essentially normal histologically. The decorin-treated wounds resembled fetal wounds in the first two trimesters.

EXAMPLE VII
25 CLONING OF HUMAN BIGLYCAN AND FIBROMODULIN cDNAs

Total cellular RNA was extracted by using guanidinium isothiocyanate (Sambrook et al., 1989) from subconfluent cultures of WI-38 human lung fibroblasts (ATCC Accession No. CCL 75; Rockville, MD) that had been exposed 30 to TGF- β 1 (3 ng/ml) for 12 hours. Total cellular RNA (1 μ g) was reverse transcribed with MoMuLV reverse transcriptase using random hexanucleotides for cDNA priming (Kawasaki, 1989). Double-stranded cDNAs encoding the full-

length coding sequences of human biglycan or human fibromodulin were generated by amplification of the reverse transcribed WI-38 RNAs using amplimers based on the published sequences of human biglycan (Fisher et al., 1989) 5 or bovine fibromodulin (Oldberg et al., 1989). For decorin, a previously described decorin cDNA was used as a template (Krusius and Ruoslahti, 1986). The PCR products were subcloned into pBluescript (Stratagene, La Jolla, CA). The identities of the resulting PCR products were verified 10 by DNA sequencing (Sanger, et al., 1977). The PCR generated biglycan clone differs from the published biglycan sequence in five bases. Two sequence differences could be reconciled by re-sequencing of clone p16 of Fisher et al. (1989), kindly provided by Dr. L Fisher. The 15 remaining differences resulted in one amino acid exchange (Lys₁₇₆ to Asn₁₇₆).

Human fibromodulin was found to be highly homologous to its bovine equivalent. DNA sequencing analysis of the 1.2 kb PCR product revealed a 1128 bp open reading frame 20 that codes for a 376 amino acid protein. The deduced protein sequence shares 92% sequence identity with the previously published bovine fibromodulin sequence. Fig. 11 shows the human fibromodulin amino acid sequence aligned with the bovine fibromodulin sequence and the sequences of 25 the other two proteoglycans used in this study.

EXAMPLE VIII

EXPRESSION VECTOR CONSTRUCTION AND FUSION PROTEIN PURIFICATION

A modified MBP sequence with a COOH-terminal factor 30 Xa-cleavage site was introduced into a vector, pQE-8 (Quiagen; Chatsworth, CA), that also encoded an affinity tag consisting of a cassette of six histidines. The pQE- 8/MBP expression vector was generated by subcloning the BglII/BamHI DNA fragment coding for MBP and a factor Xa 35 protease cleavage site into the BamHI site of pQE-8 (Fig.

12). BamHI fragments coding for the core proteins of human biglycan, decorin or fibromodulin were subsequently cloned into the resulting single BamHI site in pQE-8/MBP (Fig. 12).

5 The histidine affinity tag allowed the purification of the fusion proteins to >95% purity in a single purification step that employs a Ni-metal-chelate affinity column (Hochuli et al., 1987).

10 Biglycan, decorin, and fibromodulin were prepared according to the instructions of Qiagen (Chatsworth, CA). Briefly, recombinant bacteria were grown in LB medium containing ampicillin (100 µg/ml) and kanamycin (25 µg/ml) at 37°C to a density of OD₆₀₀ ~0.6 to 0.8. IPTG was then added to a final concentration of 2 mM and protein expression was allowed to proceed for 3 h. The bacteria were then collected by centrifugation (5000xg, 15 min) and lysed for 45 to 60 minutes in buffer A (0.1 M NaHPO₄, 0.01 M Tris, 6 M guanidinium-HCl, pH 8.0). The lysates were centrifuged for 20 min at 20,000xg. Imidazole was added to 15 the supernatants to a final concentration of 10 mM and the mixtures were loaded on Ni-NTA columns. The columns were washed with several column volumes of buffer B (0.1 M NaHPO₄, 0.01 M Tris, 8 M urea, pH 8.0) and was eluted with buffer C (buffer B adjusted to pH 5.9). Protein-containing 20 fractions were adjusted to pH 8 by adding 1 M Tris-HCl in 8 M urea. After reduction of the proteins with dithiothreitol and carboxymethylation with iodoacetamide (Charbonneau, 1989), the proteins were separated from the reagents, and buffers exchanged for the respective binding 25 30 buffers by gel filtration using PD-10 columns (Pharmacia LKB Biotechnology).

The purified MBP-fusion proteins displayed electrophoretic mobilities in SDS-PAGE compatible with the predicted amino acid sequences (Fig. 13). These proteins

were relatively soluble in physiological buffers, although some precipitation occurred during prolonged storage at 4°C.

EXAMPLE IX

5

PROTEIN IODINATION

The bacterially expressed fusion proteins were iodinated using IODO-GEN according to the manufacturer's instructions (Pierce Chemical Co.). Briefly, 50 μ l of an IODO-GEN solution (1 mg/25ml CHCl₃) were dried to the bottom 10 of a borosilicate glass tube. Protein (10-20 μ g) dissolved in iodination buffer (0.1 M NaPi, 1 mM EGTA, 150 mM NaCl, pH 7.4) and carrier-free Na¹²⁵I were added to the tube. After incubation for 12 minutes at room temperature, 200 μ l of iodination buffer and the mixture were loaded into a PD-10 15 column for radiochemical purification of the labeled protein. The specific activities and radiochemical purities of the labeled fusion proteins were calculated by determination of the picric acid precipitable radioactive protein fraction in the labeling mixture before and after 20 the purification step. The specific activities ranged from 2300 to 2800 Ci/mmol, with radiochemical purities greater than 95%. TGF- β 1, 2 AND 3 (1-5 μ g) were labeled as described above using 0.25 M NaPi, 2 M urea, pH 7.4, as iodination buffer.

25

EXAMPLE X

EQUILIBRIUM BINDING EXPERIMENTS

Solid-phase binding assays were performed incubating radiolabeled MBP-proteoglycan fusion proteins in microtiter wells coated with increasing amounts of TGF- β 1.

30

Immulon-2 microtiter wells (Dynatech; Chantilly, VA) were coated with TGF- β 1 (75 μ l, 1 μ g/ml) or other proteins dissolved in 0.1 M bicarbonate buffer, pH 9.5, overnight at

4°C. The coated wells were then flicked dry and incubated with 200 μ l of binding buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2% BSA, 0.05% Tween-20, 0.01% NaN₃) for 3 hours at 37°C to block nonspecific binding sites. Plates were 5 either used immediately or stored for future use at -20°C for up to 2 weeks. For binding assays, the blocked wells were washed once then labeled and unlabeled proteins were added in a total volume of 100 μ l binding buffer and incubated for 6 hours at 37°C if not indicated otherwise. 10 After that, the well contents were removed by aspiration and the wells were washed three times with ice-cold binding buffer. Binding to the surface-immobilized proteins was determined by counting the entire wells in a gamma counter.

Non-specific binding to the wells was less than 5% of 15 the total radioactivity added. The coating efficiency for TGF- β 1 was 58.7 \pm 0.5% (n=3), giving approximately 44 ng of TGF- β 1 per well. The coating efficiency was calculated by adding a small amount of ¹²⁵I-labeled TGF- β 1 to the coating 20 solution and determining the surface-associated radioactivity after the overnight incubation period and the subsequent washing step. All experiments were performed in duplicate or triplicate.

Radiolabeled MBP-biglycan (MBP-BG), MBP-decorin (MBP-DEC) and MBP-fibromodulin (MBP-FM) bound to TGF- β 1-coated 25 wells in a concentration-dependent manner, displaying maximum binding of 50%, 20%, and 55%, respectively (Fig. 14). Radiolabeled MBP alone did not bind to the TGF- β 1-coated wells.

The binding of the radiolabeled fusion proteins was 30 specific for TGF- β , since little to no binding was observed to immobilized NGF, EGF, insulin or platelet factor 4. MBP-FM bound slightly to immobilized TGF- β 1 precursor protein, but MBP-BG and MBP-DEC did not (Fig. 15).

Since the biglycan fusion protein, MBP-biglycan, showed high binding activity toward TGF- β 1, it was used to characterize further the proteoglycan-TGF- β 1 interactions. The binding of MBP-BG to TGF- β 1 was time- and temperature-dependent (Fig. 16). Binding increased rapidly at 37°C but very slowly at 4°C, reaching at 4°C only about 20% of the maximum binding seen at 37°C.

Unlabeled MBP-BG competed for the binding of 125 I-labeled MBP-BG to TGF- β 1 in a concentration-dependent manner (Fig. 17A). MBP-DEC and MBP-FM competed for the binding of labeled MBP-BG to TGF- β 1. They were about equally effective competitors as MBP-BG, yielding half-maximal inhibitory concentrations of about 30 to 40 nM. MBP was inactive. When purified bovine proteoglycans were used as competitors, biglycan and decorin were found to be less potent than fibromodulin; the half-maximal inhibitory concentrations were 150, 200 and 10 nM, respectively.

Data from the assays shown in Figure 17A were analyzed using the LIGAND computer program (Munson and Rodbard, 1980) to calculate dissociation constants (K_d) and maximal binding site concentrations (B_{max}) for the interaction of the proteoglycan fusion proteins with TGF- β 1. Best results were obtained for two-site binding models with K_d values ranging from 1 to 17 nM for high affinity binding sites and 47 to 200 nM for low affinity binding sites, respectively. The B_{max} values were in the range of 10^{-13} moles per well for the high affinity binding sites and 1.6×10^{-12} moles per well for the low affinity binding sites, respectively. Given a TGF- β 1 coating concentration of 1 μ g/ml, a coating volume of 75 μ l and a coated efficiency of about 60%, these values indicate a molar ratio between proteoglycan fusion protein and TGF- β 1 of one to ten for the high affinity binding site and one to one for the low affinity binding site, respectively.

Proteoglycans were also tested for their ability to bind TGF- β 2 and TGF- β 3, the other known mammalian isoforms of TGF- β . Binding of TGF- β 1, 2, and 3 to immobilized MBP-biglycan was inhibited by all three fusion proteins and all 5 three intact proteoglycans (Fig. 18). TGF- β 3 binding to MBP-BG was more effectively inhibited by decorin and biglycan than fibromodulin.

Solid-phase radioligand binding studies showed that recombinant fusion proteins containing the core protein 10 sequences of human biglycan, decorin and fibromodulin compete for binding of labeled MBP-biglycan to TGF- β 1 with similar affinities, indicating that functionally highly conserved regions of the core proteins are involved in the binding to TGF- β . The fact that bacterially produced 15 recombinant proteoglycan core proteins had similar activities to recombinant decorin produced by mammalian cells (Yamaguchi et al., 1990) and tissue-derived proteoglycans definitively established the presence of the TGF- β binding activity in the core proteins of these 20 proteoglycans.

EXAMPLE XI
CELL BINDING EXPERIMENTS

The ability of the proteoglycan fusion proteins to compete for TGF- β 1 binding to cells was tested in cell- 25 binding experiments. Cell binding experiments were performed according to Massague (1987). Briefly, subconfluent monolayers of MvLu or HepG2 cells (ATCC Accession Nos. CCL 64 and HB 8065, respectively; Rockville, MD) in 48- or 24-well cell culture dishes (Costar; 30 Cambridge, MA) maintained in DMEM containing 10% FCS were used in binding experiments. Cells were incubated with labeled TGF- β 1 in the presence or absence of unlabeled TGF- β 1 or proteoglycan fusion proteins. The cells were washed twice with ice-cold binding buffer (128 mM NaCl, 5 mM KCl,

5 mM MgSO₄, 1.2 mM CaCl₂, 50 mM HEPES, pH 7.5, 2 mg/ml BSA) and then incubated with cell binding buffer for 30 minutes at 4°C to remove endogenous receptor-associated TGF- β . Samples containing labeled and unlabeled proteins were 5 added to the wells in a total volume of 100 μ l for 48-well or 200 μ l for 24-well dishes and were incubated at 4°C with gentle agitation on a rotary platform. After incubation for 4 hours, the cells were washed three times with binding buffer. One-hundred microliters (200 μ l for 24-well 10 dishes) of solubilization buffer (25 mM HEPES, pH 7.5, 10% glycerol, 1% Triton X-100, 1 mg/ml BSA) were added to each well and incubated for 30 minutes at 4°C. Cell-associated radioactivity from triplicate samples was determined by counting a portion of the solubilized cells in a gamma 15 counter.

Whereas unlabeled TGF- β 1 competed effectively for the binding of labeled TGF- β 1 to all three types of TGF- β receptors, much higher concentrations of the proteoglycan fusion proteins were needed to compete for TGF- β 1 binding 20 to these cells (Fig. 19A). Half-maximal competition was achieved by fusion protein concentrations averaging about 3 μ M.

EXAMPLE XII
CROSS-LINKING OF TGF- β TO RECEPTORS

25 For receptor cross-linking, cells were grown in 24-well culture dishes and were processed as described above. After incubation with labeled and unlabeled ligands, the cells were washed once with binding buffer and three times with binding buffer without BSA. Then 100 μ l of binding 30 buffer (without BSA) containing disuccinimidyl suberate (final concentration 0.2 mM) were added, and the cells were incubated for 15 min at 4°C. After the cross-linking reaction, the cells were lysed in 100 μ l of solubilization buffer (125 mM NaCl, 10 mM Tris, pH 7, 1 mM EDTA, 1% Triton

X-100, 10 μ g/ml leupeptin, 10 μ g/ml antipain, 50 mg/ml aprotinin, 100 μ g/ml benzamidine-HCl, 10 μ g/ml pepstatin). The lysates were mixed with sample buffer and analyzed by SDS-PAGE under reducing conditions using precast 4-12% Novex gels. After electrophoresis the gels were dried and exposed to XAR-100 x-ray film for several days at -70°C.

Cross-linking experiments revealed that TGF- β 1 binding to the type I and type III receptors was affected more by all three proteoglycan fusion proteins than binding to the type II receptors, which was essentially unchanged (Fig. 19B). Laser densitometry analyses of the respective autoradiograms showed that the binding of labeled TGF- β 1 to type I and III receptors was decreased by approximately 25% or 50%, respectively, at the proteoglycan concentration used.

Competition was most effective for TGF- β binding to the type I and type III TGF- β receptors, perhaps because these receptors have a lower affinity for TGF- β than does the type II receptor. While the affinities of the proteoglycans for the TGF- β are much lower than those of any of the receptors, all of the proteoglycans are abundant in tissues potentially making up in concentration what they may lack in affinity. Moreover, the experimental conditions used in the cell binding experiments do not favor the binding of TGF- β to proteoglycans.

Although the invention has been described with reference to the presently-preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

We claim:

1. A method for the prevention or reduction of scarring comprising administering decorin or a functional equivalent of decorin to a wound.
- 5 2. The method of claim 1, wherein said functional equivalent is biglycan.
3. The method of claim 1, wherein said functional equivalent is fibromodulin.
4. The method of claim 1, wherein said scarring is
10 dermal scarring.
5. A pharmaceutical composition comprising decorin or its functional equivalent and a pharmaceutically acceptable carrier.
6. The pharmaceutical composition of claim 5,
15 wherein said functional equivalent is biglycan.
7. The pharmaceutical composition of claim 5,
wherein said functional equivalent is fibromodulin.
8. The pharmaceutical composition of claim 5,
wherein said pharmaceutically acceptable carrier is
20 hyaluronic acid.
9. The pharmaceutical composition of claim 5,
wherein said composition further comprises an RGD-containing polypeptide attached to a biodegradable polymer.

10. A method of treating a pathology caused by a TGF-
B regulated activity comprising contacting the TGF-B with
a purified polypeptide, wherein the polypeptide comprises
a TGF-B binding domain of a protein and wherein the protein
5 is characterized by a leucine-rich repeat of about 24 amino
acids, whereby the pathology causing activity is prevented
or reduced.

11. The method of claim 10, wherein said protein is
decorin.

10 12. The method of claim 10, wherein said protein is
biglycan.

13. The method of claim 10, wherein said protein is
fibromodulin.

14. The method of claim 10, wherein said pathology is
15 rheumatoid arthritis, glomerulonephritis, arteriosclerosis,
adult respiratory distress syndrome, cirrhosis of the
liver, fibrotic cancer, fibrosis of the lungs, post-
myocardial infarction, cardiac fibrosis, post-angioplasty
restenosis, renal interstitial fibrosis or dermal fibrotic
20 conditions.

15. The method of claim 14, wherein said pathology is
glomerulonephritis.

1/20

FIG. IA

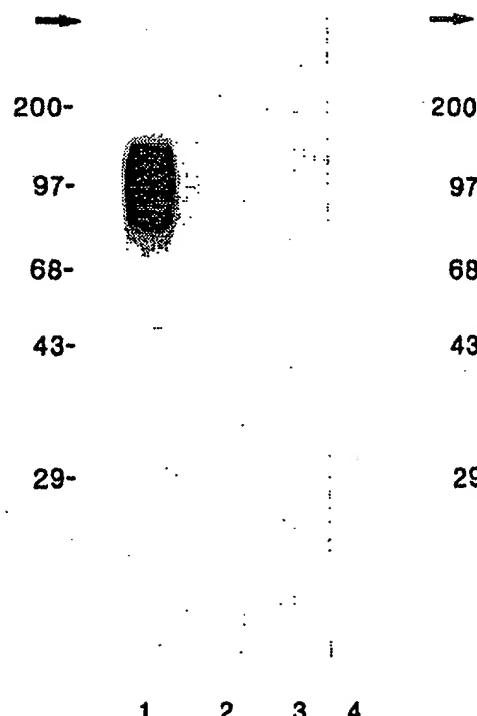
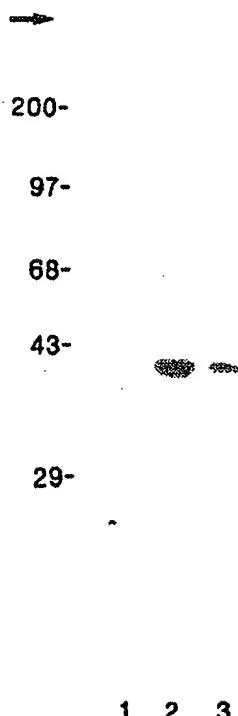


FIG. IB



2 / 20

FIG. 2A

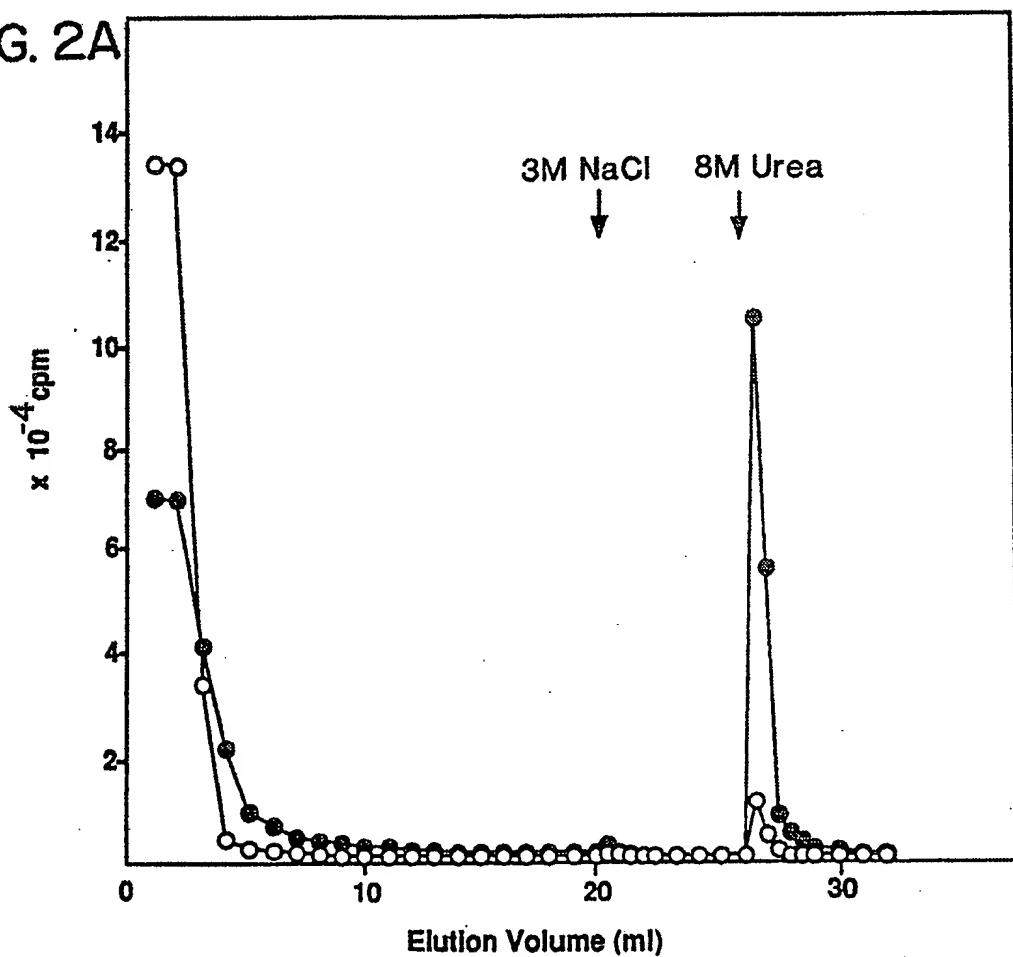
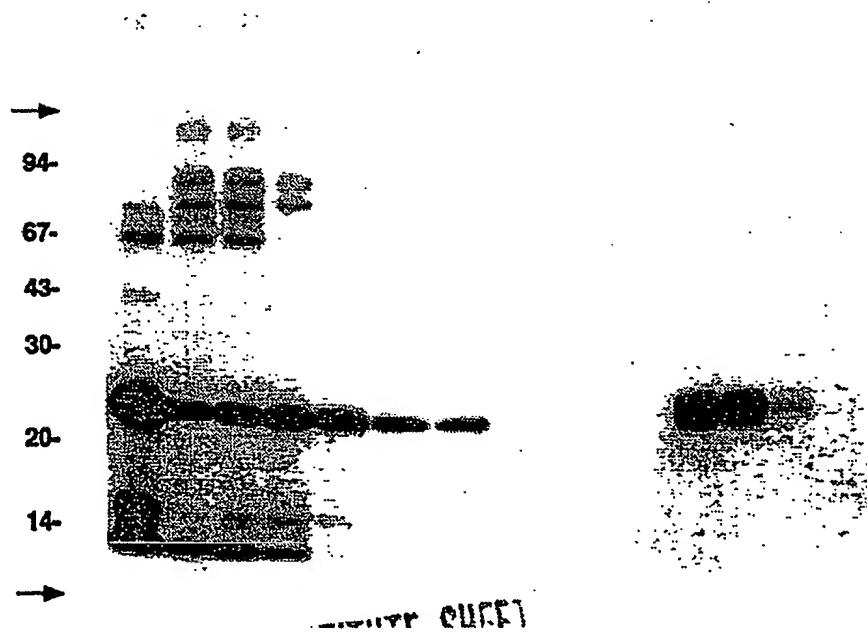


FIG. 2B

1 2 3 4 5 6 7 8 9 10 11 12 13 14



3/20

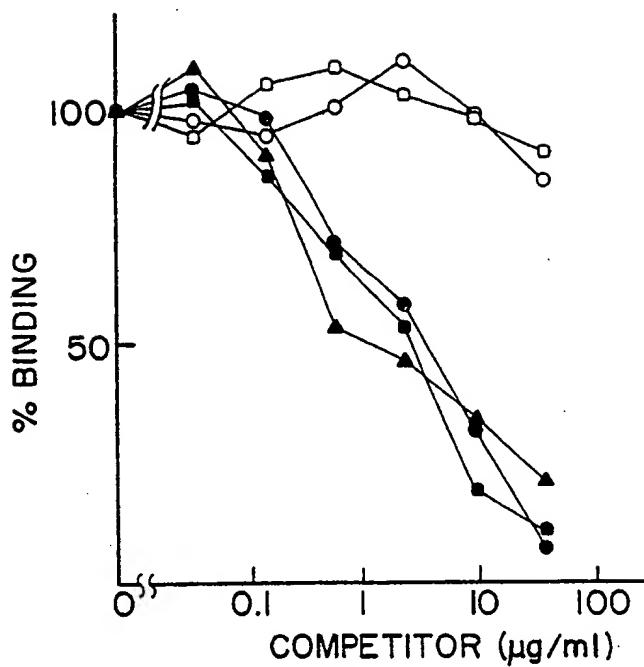


FIG. 3A

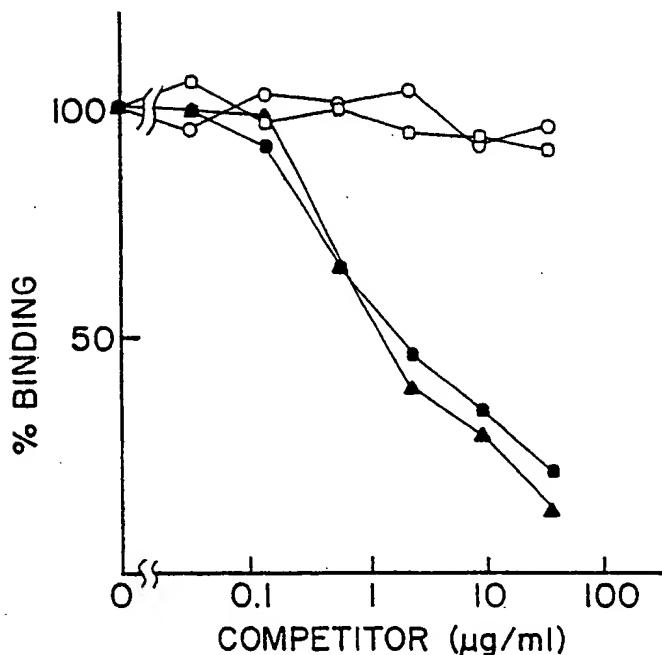


FIG. 3B

4 / 20

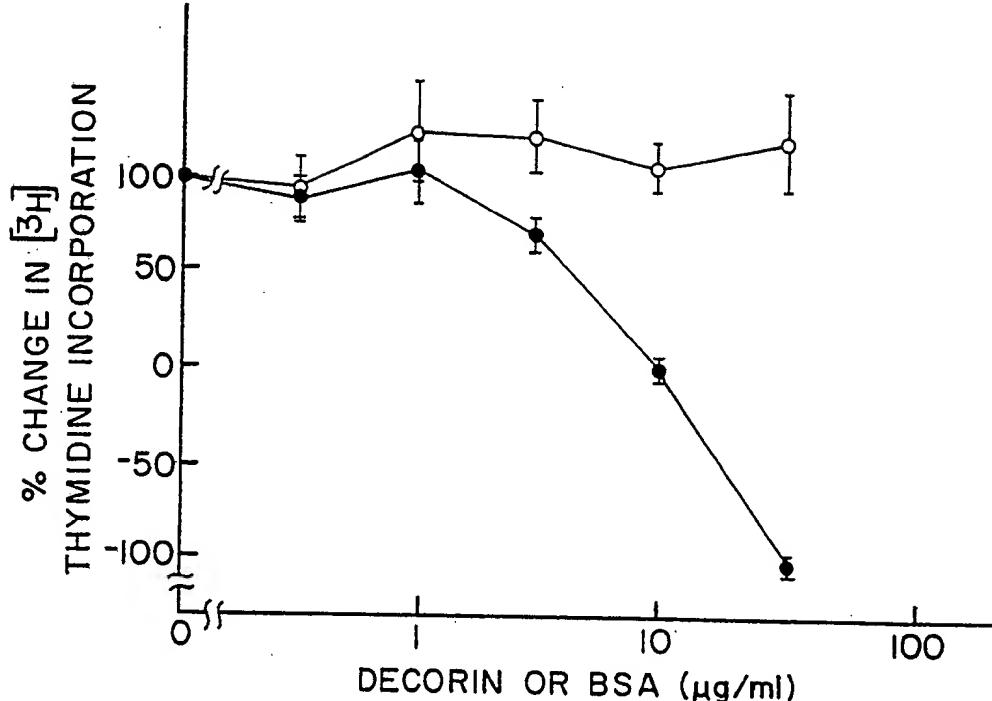


FIG. 4A

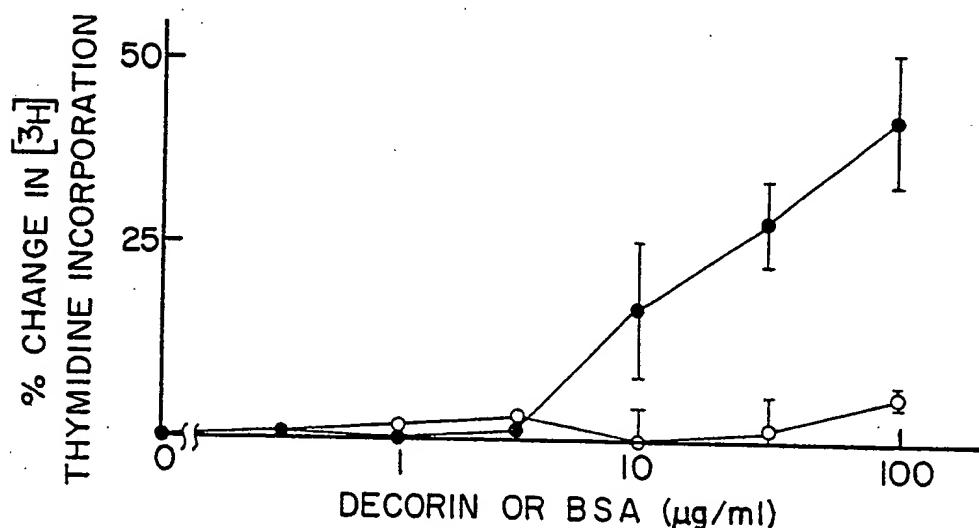


FIG. 4B

5/20

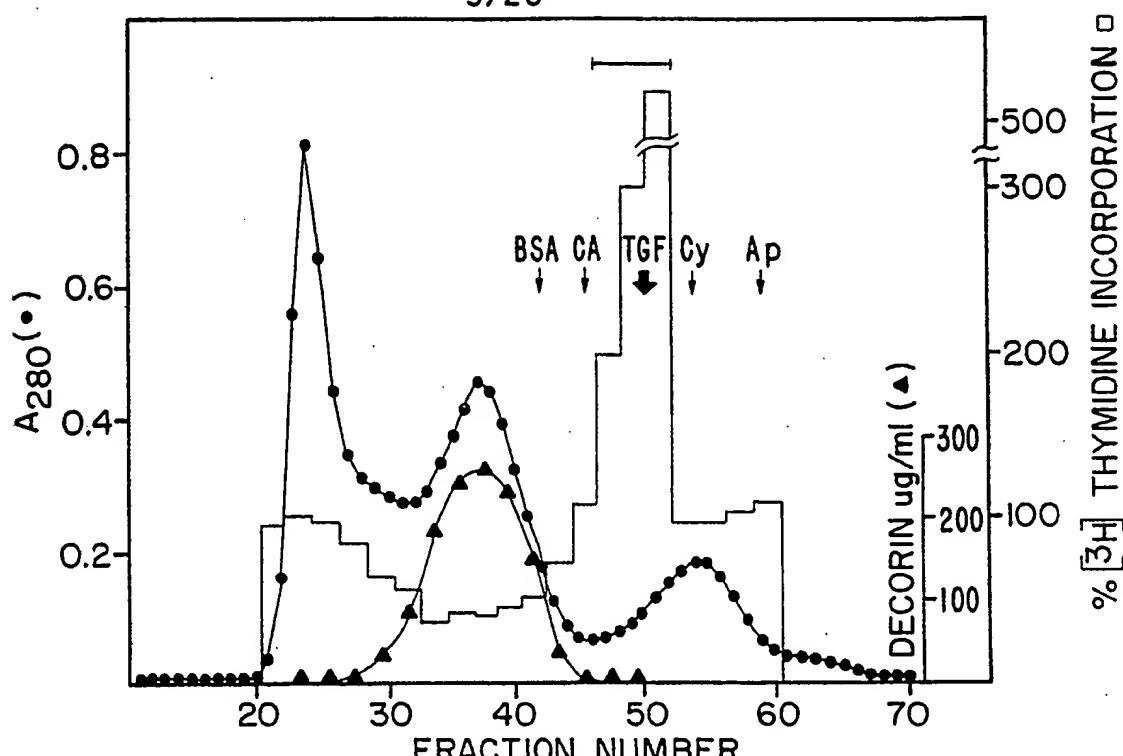


FIG. 5A

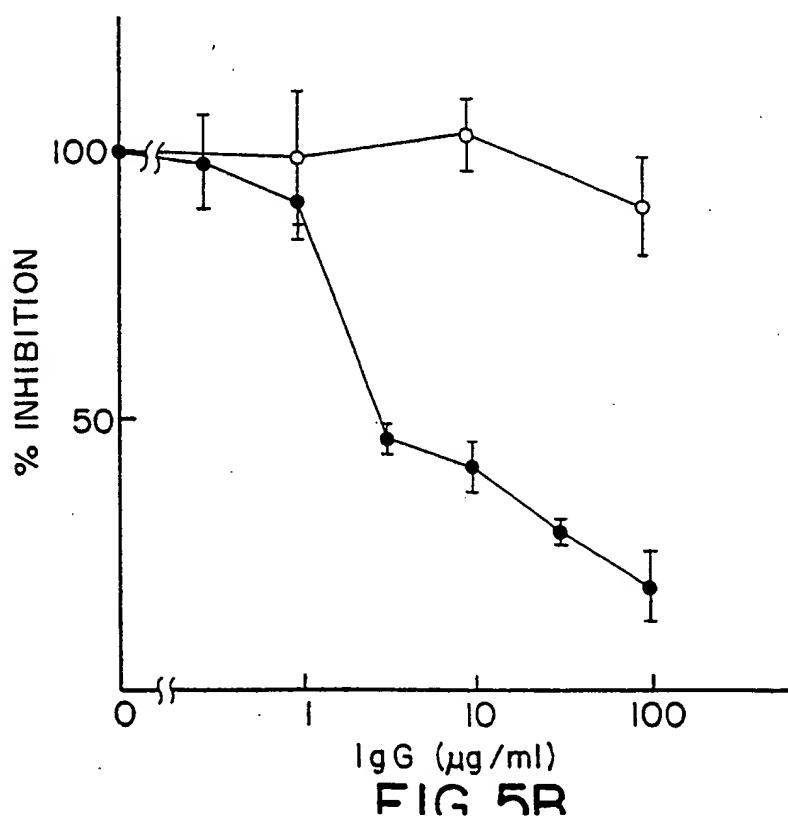


FIG. 5R

6/20

FIG. 6A

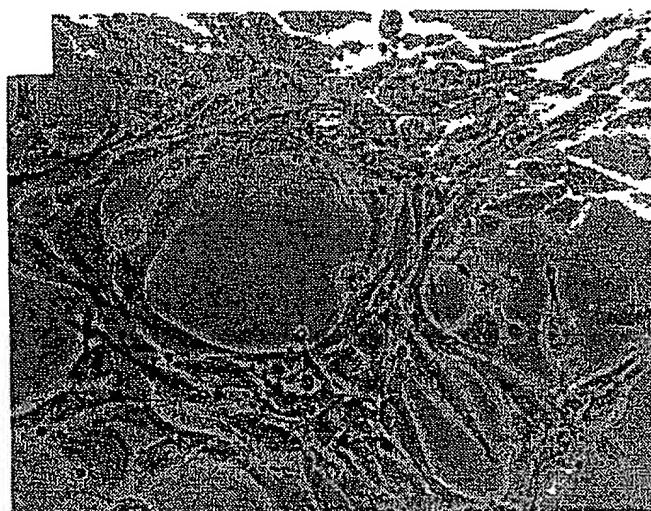


FIG. 6B

7/20

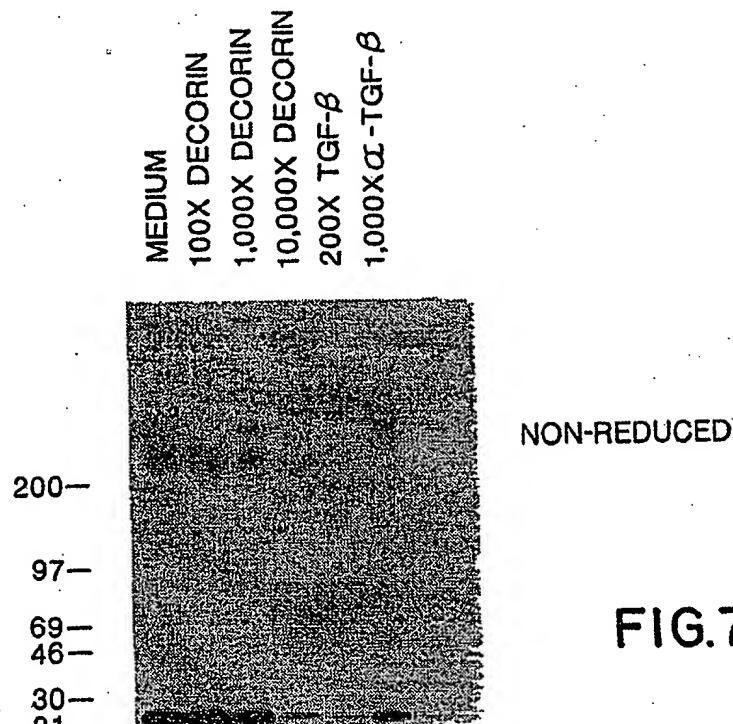


FIG.7a

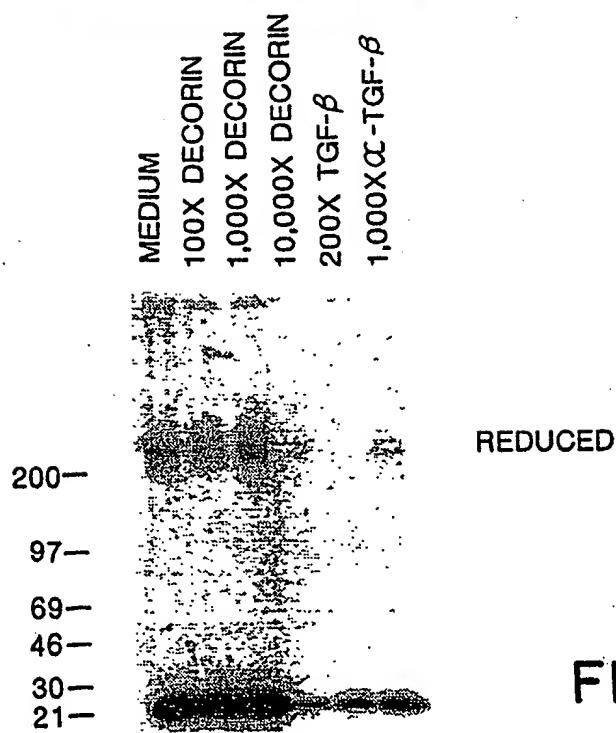


FIG.7b

8/20

FIG. 8a

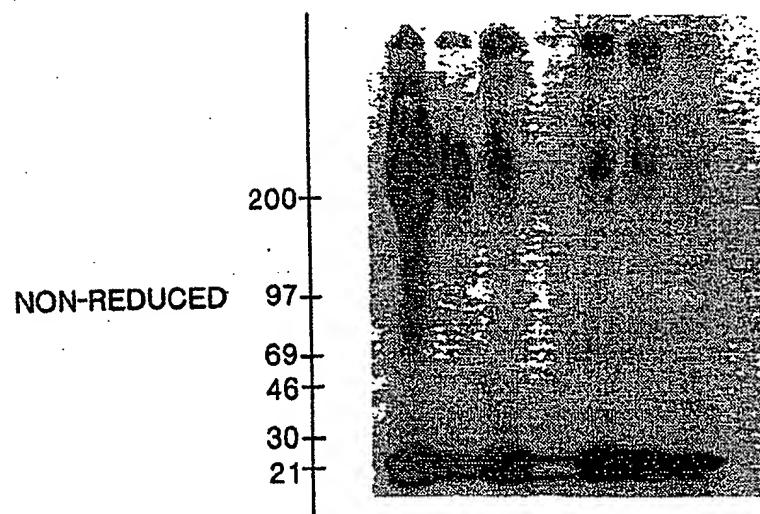
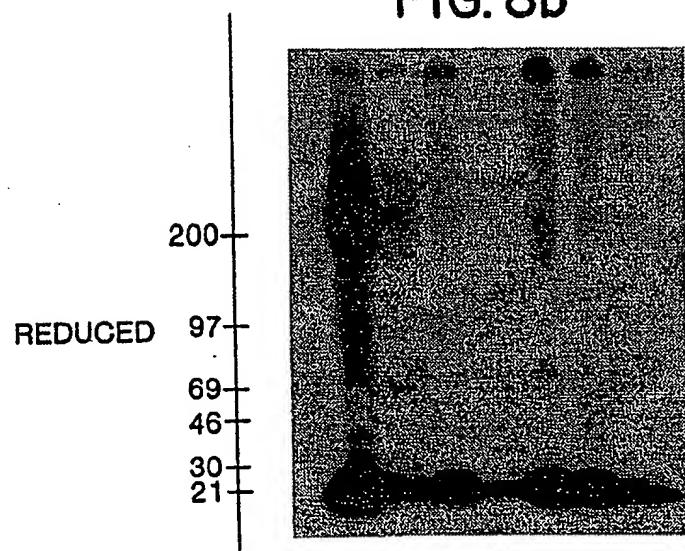


FIG. 8b



HepG2
HepG2+TGF- β
MG63
MG63+TGF- β
+100X DC-13
+1,000X DC-13
+10,000X DC-13

9/20

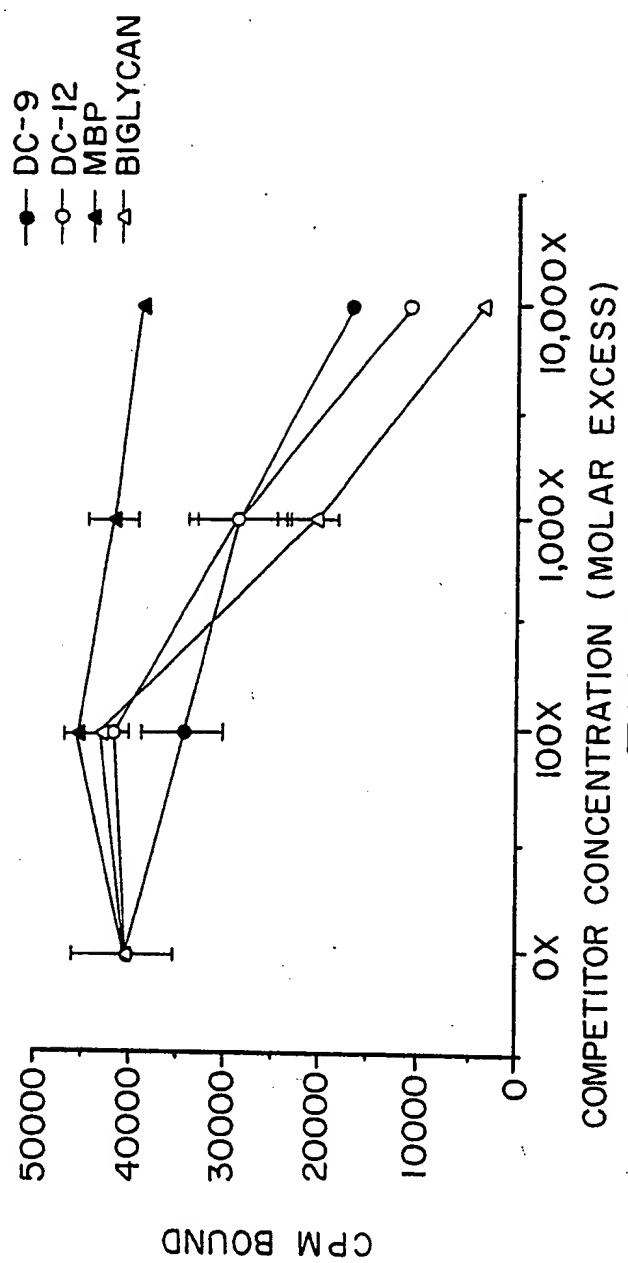
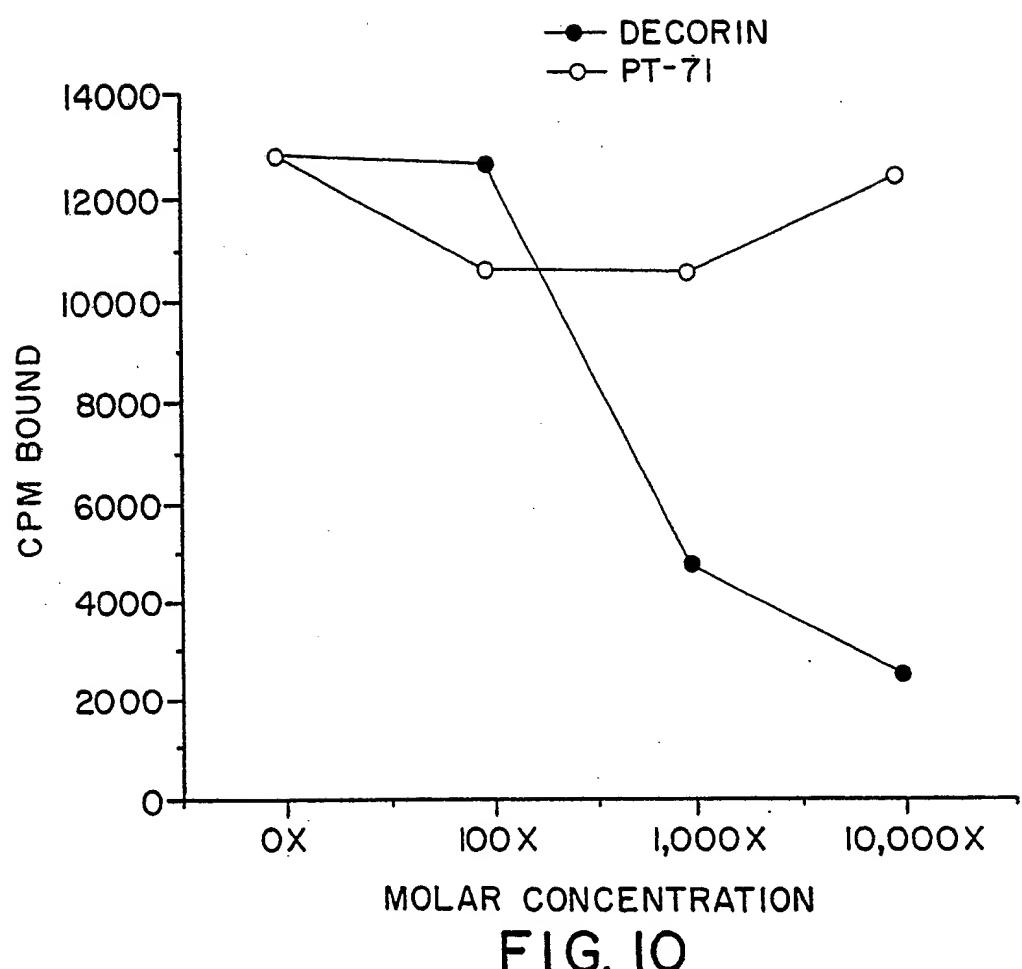


FIG. 9

10/20



11/20

hum. FM [M] QWAS L L L A G [L] F S L S Q A Q Y E D D P H W W F H Y L R S Q Q S T Y Y D P Y D P Y P Y E T Y 50
 bov. FM [M] QWAS I L L A G [L] C S L S W A Q Y E E D S H W W F Q F L R N Q Q S T Y D D P Y D P Y P Y E P Y 50
 hum. BG [M] - WPL W R L V S - L L A L S Q A L P F E Q R G F W - D F T L D D - - - - - 6 P F M M N D E 39
 hum. DEC [M] - KAT I I L L - L A Q V S W A G P F Q Q R G L F - D F M L E D - - - - - 31

hum. FM [E] P Y P Y G V D E G P A Y T Y G S P S P P D P R D C P Q E C D C P P N F L T A M Y C D N R N L K Y L 100
 bov. FM [E] P Y P T G - E E G P A Y A Y G S P P Q P E P R D C P Q E C D C P P N F P T A M Y C D N R N L K Y L 99
 hum. BG [E] A S G A D T S G V L D - P D S V T P T Y S A M - C P F G C H C - - H L R V V Q C S D L G L K S V P 85
 hum. DEC [E] A S S I G - P E V P D - D R D F E P S L G P V - C P F R C Q C - - H L R V V Q C S D L G L D K V P 76

hum. FM P F V P S R M K Y V Y F Q N N Q I T S I Q E G V F D N A T G L L W I A L H G N Q I T S D K V G R K V 150
 bov. FM P F V P S R M K Y V Y F Q N N Q I S S I Q E G V F D N A T G L L W I A L H G N Q I T S D K V G K K V 149
 hum. BG K E I S P D T T L L D L Q N N D I S E L R K D D F K G L Q H L Y A L V L V N N K I S - - K I H E K A 133
 hum. DEC K D L P P D T T L L D L Q N N K I T E I K D G D F K N L K N L H A L I L V N N K I S - - K V S P G A 124

hum. FM F S K L R H L E R [Y] L D H [N] T R M P G P L P R S L R E L H L D H H N Q I S R V P N N A L E G L E 200
 bov. FM F S K L R H L E R [Y] L D H [N] T R I P S P L P R S L R E L H L D H H N Q I S R V P N N A L E G L E 199
 hum. BG F S P L R N V Q K L Y I S K N H L V E I P P N L P S S L V E L R I H D N R I R K V P N G V F S G L R 183
 hum. DEC F T P L V K L E R [Y] L S K N Q L K E L P E K M P K T L Q E L R A H E N E I T K V R K V T F N G L N 174

FIG. IIIA

12/20

hum. FM N L T A L Y L Q H D E I Q E V G - - - S S M R G L R S L I L D L S Y N H L R K V P D G L P S A L E 247
 bov. FM N L T A L Y L H H N E I Q E V G - - - S S M K G L R S L I L D L S Y N H L R K V P D G L P S A L E 246
 hum. BG N M N C I E M G G N P L E N S G F E P G A F D G L K - L N Y L R I S E A K L T G I P K D L P E T L N 232
 hum. DEC Q M I V I E L G T N P L K S S G I E N G A F Q G M K K L S Y I R I A D T N I T S I P Q G L P P S L T 224

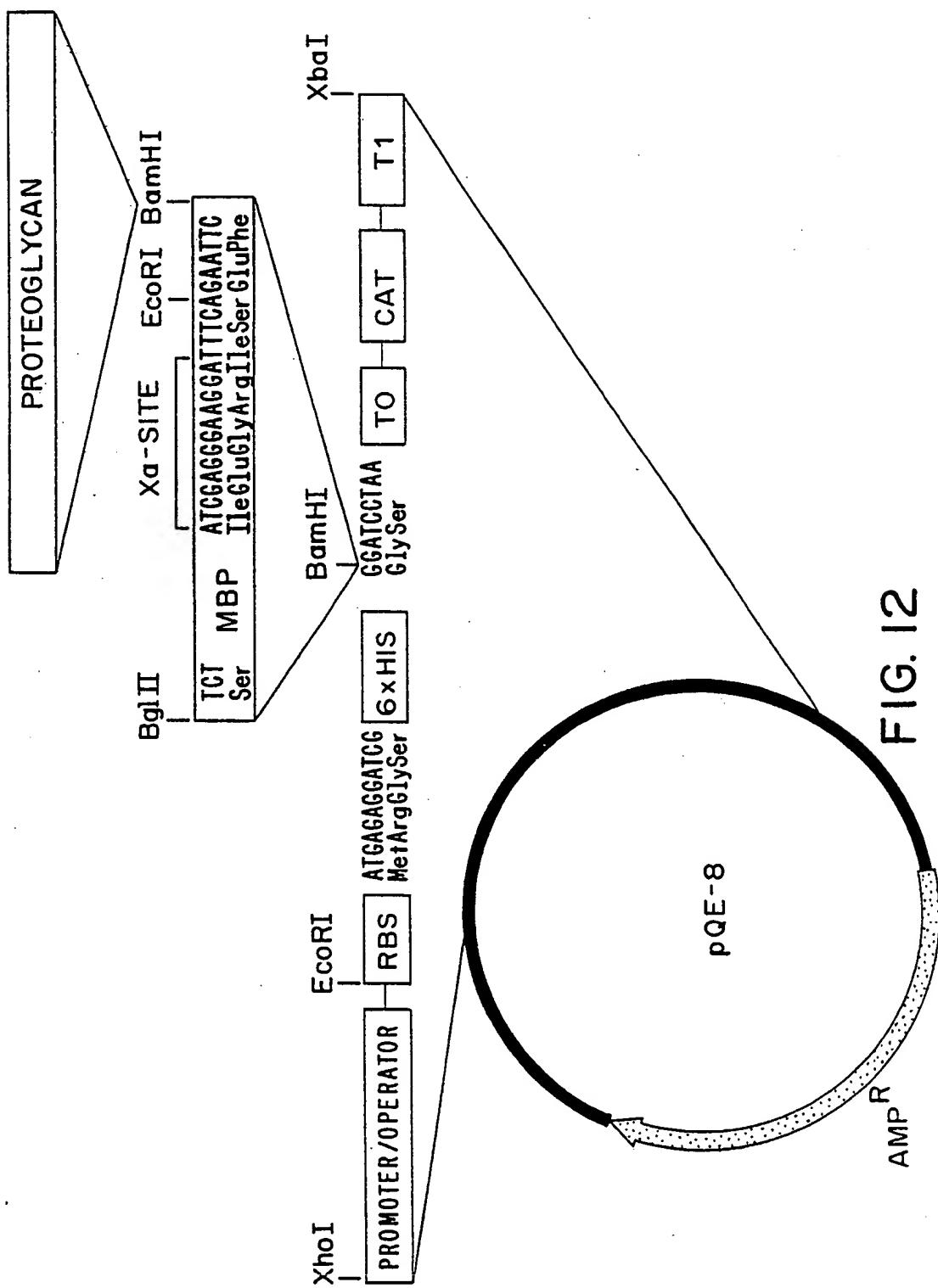
hum. FM Q L Y M E H N N V Y T V P D S Y F R G A P K L L Y V R L S H N S L T N N G L A S N T F N S S - S L L 296
 bov. FM Q L Y L E H N N V F S V P D S Y F R G S P K L L Y V R L S H N S L T N N G L A S N T F N S S - S L L 295
 hum. BG E L H L D H N K I Q A I E L E D L L R Y S K L Y R L G L G H N Q I R M I E N G S L S F L P T L R E L 282
 hum. DEC E L H L D G N K I S R V D A A S L K G L N N L A K L G L S F N S I S A V D N G S L A N T P H L R E L 274

hum. FM E L D L S - Y N Q L Q K I P P V N T N L E N L Y L Q G N R T N E F S I S S F C T V V D V V N F S K L 345
 bov. FM E L D L S - Y N Q L Q K I P P V S T N L E N L Y L Q G N R I N E F S I S S F C T V V D V M N F S K L 344
 hum. BG H L D N N K L A R V P S G L P D L K L L Q V V Y L H S N N I T K V G V N D F C P M G F G V K R A Y Y 332
 hum. DEC H L D N N K L T R V P G G L A E H K Y I Q V V Y L H N N I S V V G S S D F C P P G H N T K K A S Y 324

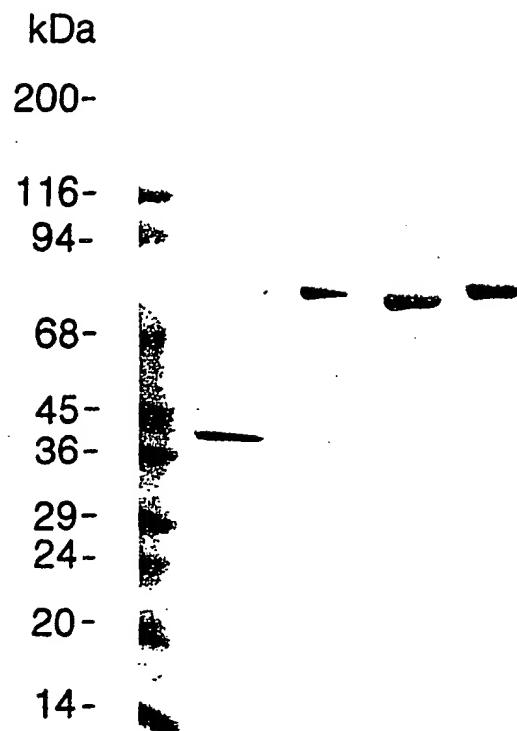
hum. FM Q V V R L D G N E I K R S A M P A D A P L C L - - R L A S L I E I - - - 376
 bov. FM Q V Q R L D G N E I K R S A M P A D A P L C L - - R L A S L I E I - - - 375
 hum. BG N G I S L F N N P V P Y W E V Q P A T F R C V T D R L A I Q F G N Y K K 368
 hum. DEC S G V S L F S N P V Q Y W E I Q P S T F R C V Y V R S A I Q L G N Y K - 359

FIG. IIB

13/20

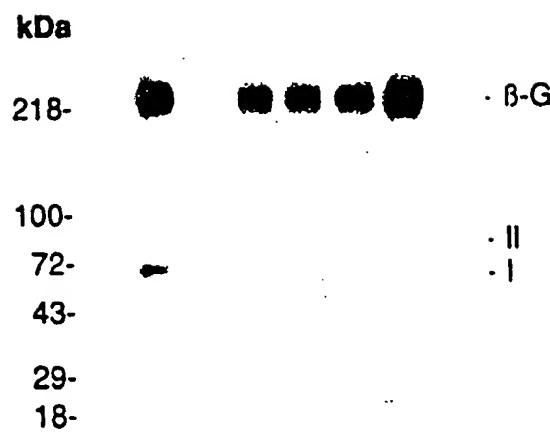


14/20
FIG. 13



A B C D

FIG.19B



15/20

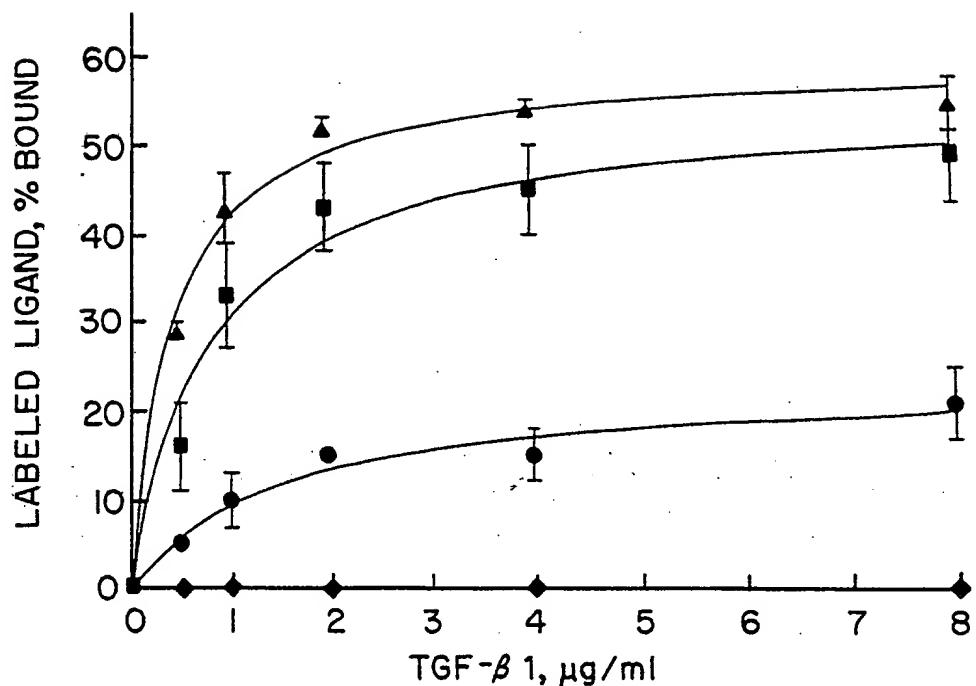
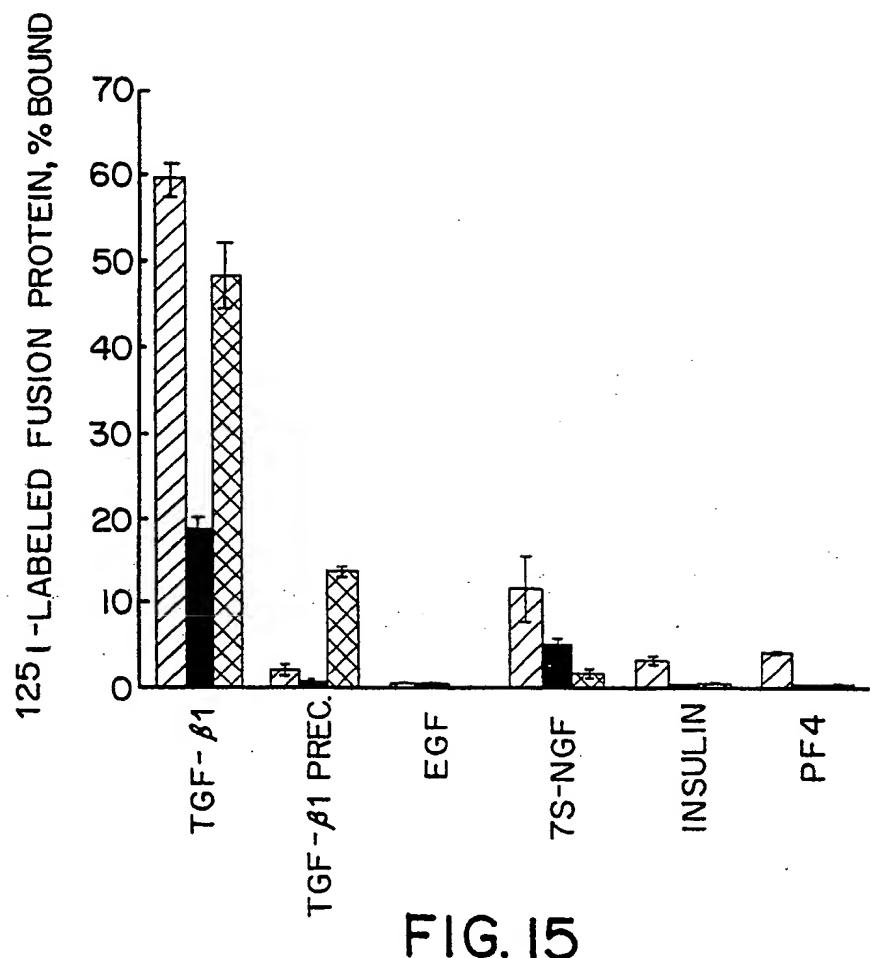


FIG. 14

16 / 20



17/20

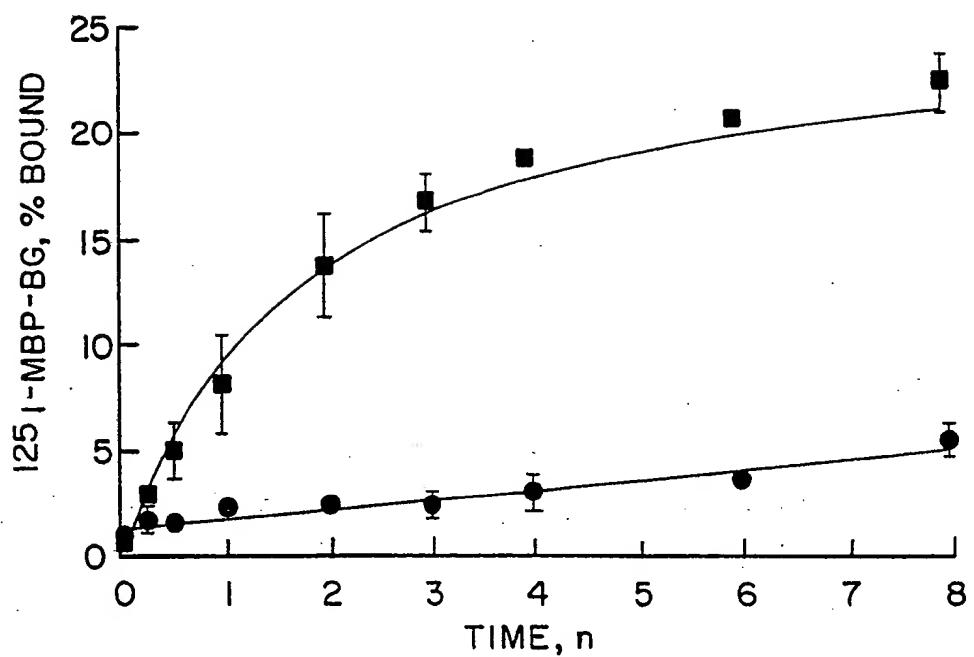


FIG. 16

18/20

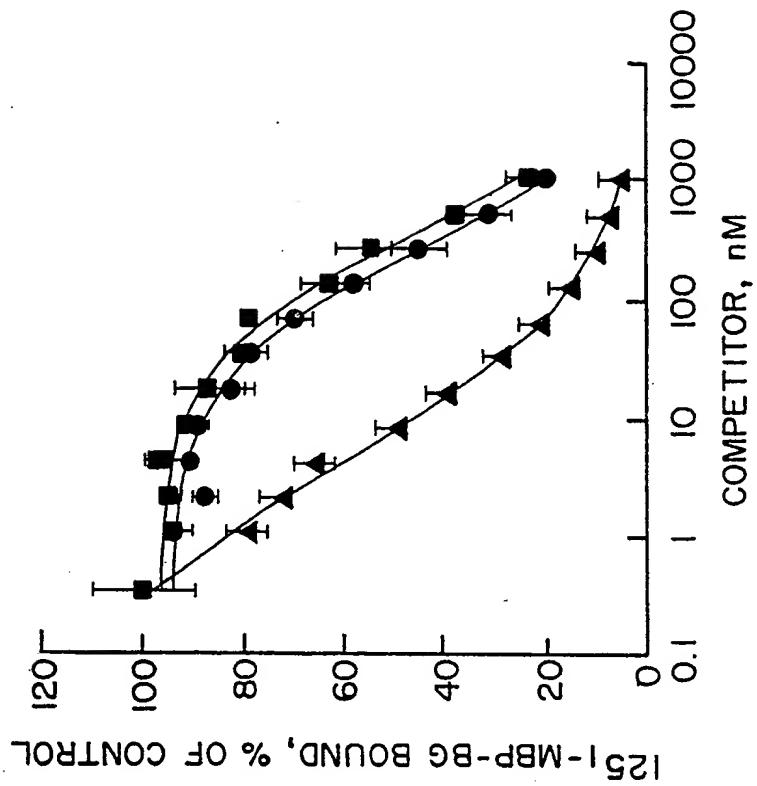


FIG. 17B

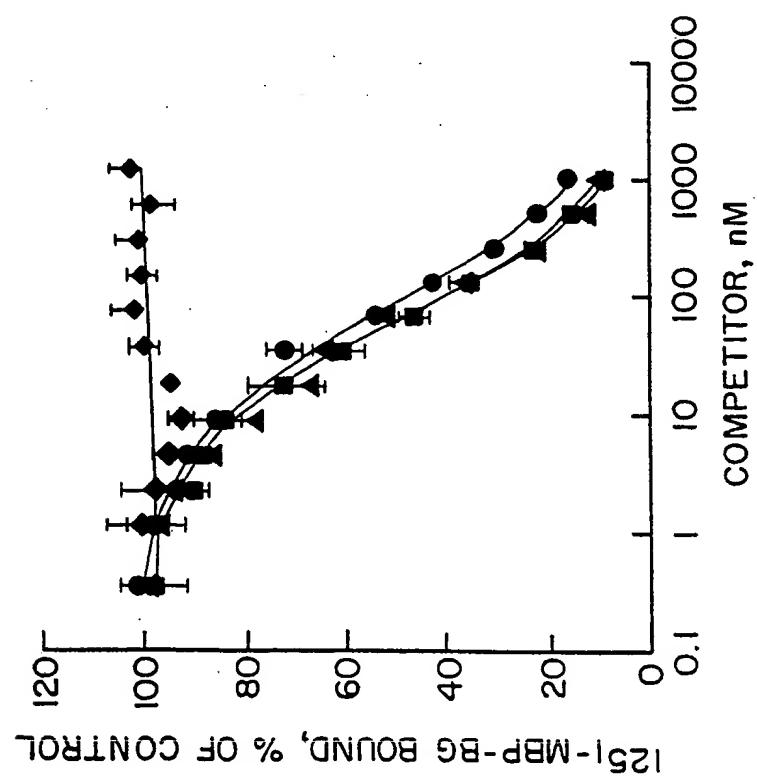
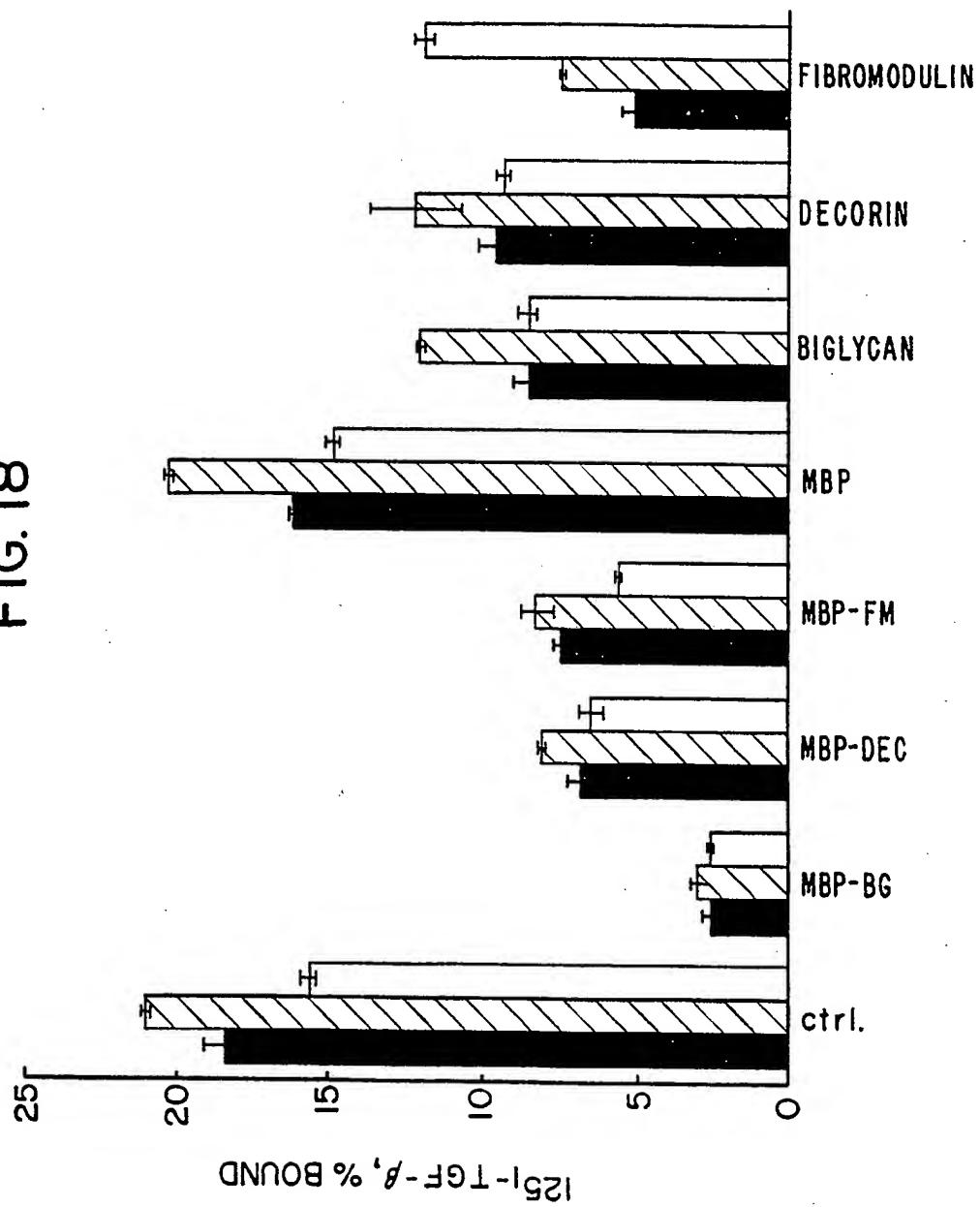


FIG. 17A

19 / 20

FIG. 18



20/20

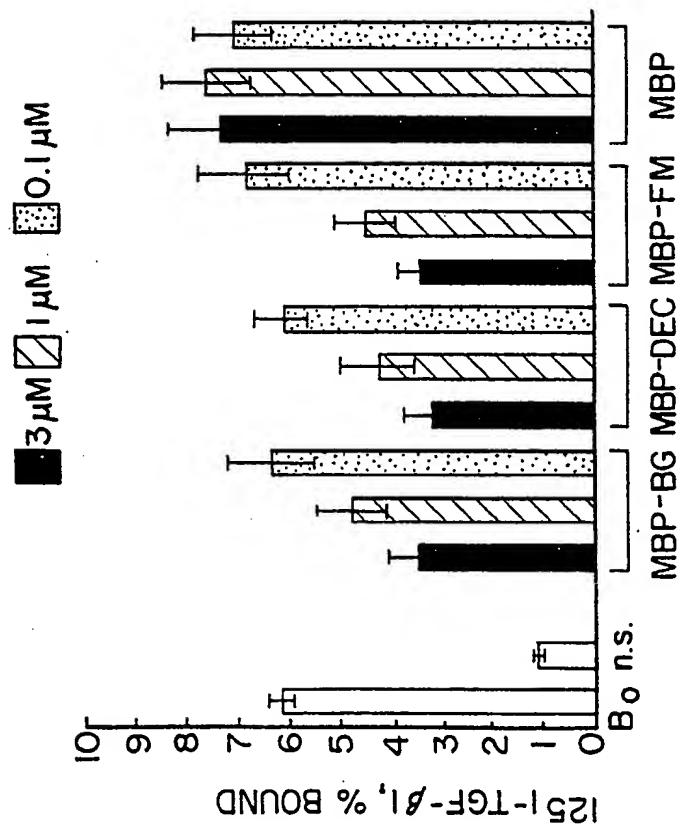


FIG. 19A

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.C1. 5 A61K37/02; //C07K13/00, C12N15/12, C12N15/62

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols		
Int.C1. 5	C07K	A61K	C12N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	WO,A,9 110 727 (LA JOLLA CANCER RESEARCH FOUNDATION) 25 July 1991 see claims 1-4,8-11,27-34 ---	1-7, 10-15
X	EP,A,0 349 480 (LA JOLLA CANCER RESEARCH FOUNDATION) 3 January 1990 see page 2, line 17 - line 28; claims 6-8 ---	1-7, 10-15
X	NATURE. vol. 346, 19 July 1990, LONDON GB pages 281 - 284 Y. YAMAGUCHI ET AL. 'NEGATIVE REGULATION OF TRANSFORMING GROWTH FACTOR-BETA BY THE PROTEOGLYCAN DECORIN.' see page 284, left column, line 8 - line 52 ---	1-7, 10-15
Y		8,9 -/-

¹⁰ Special categories of cited documents :¹⁰¹¹ "A" document defining the general state of the art which is not considered to be of particular relevance¹² "E" earlier document but published on or after the international filing date¹³ "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)¹⁴ "O" document referring to an oral disclosure, use, exhibition or other means¹⁵ "P" document published prior to the international filing date but later than the priority date claimed¹⁰ "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention¹¹ "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step¹² "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art¹³ "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

26 FEBRUARY 1993

Date of Mailing of this International Search Report

15.03.93

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

RYCKEBOSCH A.O.

III. DOCUMENTS CONSIDERED TO BE RELEVANT		(CONTINUED FROM THE SECOND SHEET)
Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,9 006 767 (LA JOLLA CANCER RESEARCH FOUNDATION) 28 June 1990 see claims 1-7,18-21 ---	8,9
P,X	THE JOURNAL OF CLINICAL INVESTIGATION vol. 90, no. 1, July 1992, NEW YORK, N.Y., US pages 1 - 7 W.A. BORDER ET AL. 'TRANSFORMING GROWTH FACTOR-BETA IN DISEASE: THE DARK SIDE OF TISSUE REPAIR.' see the whole document ---	1-15
P,X	KIDNEY INTERNATIONAL vol. 41, 1992, NEW YORK, N.Y., US pages 566 - 570 W.A. BORDER ET AL. 'ANTAGONISTS OF TRANSFORMING GROWTH FACTOR-BETA: A NOVEL APPROACH TO TREATMENT OF GLOMERULONEPHRITIS AND PREVENTION OF GLOMERULOSCLEROSIS.' see the whole document -----	1-15

INTERNATIONAL SEARCH REPORT

1. International application no.

PCT/US92/09871

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 1-4 and 10-15 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

US 9209871
SA 67422

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 26/02/93

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO-A-9110727	25-07-91	None			
EP-A-0349480	03-01-90	AU-A- 3865389 JP-T- 3503846 WO-A- 9000194		23-01-90 29-08-91 11-01-90	
WO-A-9006767	28-06-90	AU-B- 628910 AU-A- 4828590 CA-A- 2006060 EP-A- 0449973		24-09-92 10-07-90 20-06-90 09-10-91	